UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

DEPARTAMENTO DE QUÍMICA FARMACÊUTICA E TERAPÊUTICA

NEW TOOLS FOR CHEMICAL MODIFICATION OF PROTEINS

Pedro Miguel Saraiva Duarte Cal

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List of Publications

Papers in International Scientific Periodicals with Referees

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- "Targeting Cancer Cells with Folic Acid-Iminoboronate Fluorescent Conjugates", Pedro M. S. D. Cal, Raquel F. M. Frade, Vijay Chudasama, Carlos Cordeiro, Stephen Caddick and Pedro M. P. Gois, *Chemical Communications*, **2014**, 50, 5261-5263 - 2014 Emerging Investigators Themed collection (DOI: 10.1039/C3CC47534D)
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Abbreviations

ABBA	Acetylbenzeneboronic acid
ACN	Acetonitrile
ADC	Antibody-drug conjugate
Ala	Alanine (A)
Arg	Arginine (R)
Asn	Asparagine (N)
Asp	Aspartic Acid (D)
ATHA	Acyltrifluoroborate-hydroxylamine
BA	Boronic acid
BODIPY	Boron-dipyrromethene
BSA	Bovine serum albumin
CBT	Cyanobenzothiazole
Cbz	Carboxybenzyl (Z)
CuAAC	Copper catalyzed azide-alkyne cycloaddition
Cys	Cysteine (C)
DBU	1,8-Diazabicycloundec-7-ene
DCC	*N,N'*-dicyclohexylcarbodiimide
DCM	Dichloromethane
DCU	Dicyclohexylurea
Dha	Dihydroalanine
DMAP	*N,N*-dimethylpyridin-4-amine
DIPEA	*N,N*-Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDA-FA	*N*-(2-aminoethyl)folic acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EMA	European medicines agency
EPL	Expressed protein ligation
ESI	Electrospray ionization
EtOAc	Ethyl acetate
FA	Folic acid
FBBA	Formylbenzeneboronic acid

FDA	Food and drug administration
FTIC	Fluorescein-5-isothiocyanate
FTICR	Fourier transform ion cyclotron resonance
GFP	Green fluorescent protein
Gln	Glutamine (Q)
Glu	Glutamic Acid (E)
GST	Glutathione S-transferase
HIF*α*	Subunit *α* of the hypoxia-inducible factor
His	Histidine (H)
HIV	Human immunodeficiency virus
HMPT	Tris(dimethylamino)phosphine
HRMS	High resolution mass spectrometry
HSA	Human serum albumin
ICT	Isocyanate
iEDDA	Inverse electron-demand Diels-Alder
Ile	Isoleucine (I)
ITC	Isothiocyanate
KAHA	*α*-Ketoacid-hydroxylamine
KLH	Keyhole limpet hemocyanin
KPL	Kinetically-controlled protein labeling
Leu	Leucine (L)
LPS	Lipopolysaccharides
Lys	Lysine (K)
Lyz	Lysozyme
Met	Methionine (M)
MS	Mass spectrometry
MSH	*O*-mesitylenesulfonylhydroxylamine
NAA	Natural amino acids
NCL	Native chemical ligation
NED	Normal electron demand
NHS	*N*-hydroxysuccinimide
NIR	Near-infrared
NMR	Nuclear magnetic ressonance
NNAA	Non-natural amino acids
PEG	Polyethyleneglycol

PET	Positron emission tomography
Phe	Phenylalanine (F)
PMSF	Phenylmethylsulfonylfluoride
PPTS	Pyridinium *p*-toluenesulfonate
Pro	Proline (P)
PTM	Post-translational modifications
PTS	Protein-*trans* splicing
RITC	Rhodamine-B-isothiocyanate
RNA	Ribonucleic acid
r.t.	Room temperature
Sac	*S*-allylcysteine
SBL	Substilisin protease of *Bacillus lentus*
Ser	Serine (S)
S_NAr	Nucleophilic aromatic substitution
SPAAC	Strain promoted azide-alkyne cycloaddition
SPANOC	Strain promoted azide-nitrile oxide cycloaddition
SPECT	Single photon emission computed tomography
SPPS	Solid-phase peptide synthesis
SST	Somatostatin
STL	Serine and threonine ligation
TCEP	Tris(2-carboxyethyl)phosphine
TEA	Triethylamine
TEC	Thiol-Ene Coupling
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
THP	Tetrahydropyranyl
Thr	Threonine (T)
TLC	Thin layer chromatography
TNB	5-Thio-2-nitrobenzoic acid
TRH	Thyrotropin-releasing hormone
Trp	Tryptophan (W)
TYC	Thiol-yne coupling
Tyr	Tyrosine (Y)
UV	Ultraviolet
Val	Valine (V)

Abstract

This thesis falls within the field of chemical biology, describing a selective modification (with controlled reversibility) of primary amines of biomolecules.

Selective conjugation of species to the surface of biomolecules is extremely important in that it allows an endless number of biological applications, leading to modify the properties of biomolecules. As examples of these modifications, the mimicry of post-translational modifications of proteins, artificial glycosylation and immobilization of biomolecules to certain surfaces, incorporation of molecules for bioimaging, enhancement of pharmacokinetic properties of biomolecules and drug-delivery possibilities.

Based on published literature, a selective modification (with controlled reversibility) of primary amines on the surface of native proteins and other biomolecules was found and optimized. Its theoretical foundations were elucidated with DFT calculations and its reversibility proven with a select set of endogenous molecules.

After the above-mentioned discovery and optimized conjugation, various synthetic modifications have been made to induce specific chemical properties to the molecule, keeping the core structure that allows conjugation. Several compounds with different properties were synthesized and important biological studies were performed. Particularly, a study from which it was concluded that these iminoboronates are stable enough to go through cellular internalization and that the conjugation of a cytotoxic drug with a biomolecule can possibly increase the selectivity of the internalization of the drug. The study of intracellular reversibility of the conjugate is currently in progress through the development of an "ON-OFF" probe that is specific for this type of conjugation.

Nevertheless, in this work it was found that the conjugation system has a deficient reversibility, and for that reason experiments were initiated (ongoing work) to improve this bioconjugation to the point that a broader number of biological applications can be applied.

Keywords: Lysine functionalization, boronic acids, Iminoboronates, reversible

Resumo

A tese apresentada insere-se no campo de química biológica, descrevendo uma modificação selectiva (e com reversibilidade controlada) de aminas primárias em biomoléculas.

A conjugação selectiva de espécies à superfície de biomoléculas é extremamente importante na medida em que permite um infindável número de aplicações biológicas, podendo até modificar as propriedades inerentes da biomolécula. Como exemplos destas modificações, salientam-se a mimetização de modificações pos-translaccionais de proteínas, a glicosilação artificial e a imobilização a determinadas superfícies de biomoléculas, a incorporação de moléculas para bioimaging, a alteração de propriedades farmacocinéticas de biomoléculas e a possibilidade de distribuição selectiva de compostos com propriedades farmacológicas.

Baseado na literatura publicada, uma modficação selectiva e com reversibilidade controlada de aminas primárias à superfície de proteínas naturadas e outras biomoléculas foi encontrada e optimizada. As suas fundamentações teóricas foram elucidadas com um estudo de cálculos de DFT e a sua reversibilidade comprovada utilizando um conjunto selecto de moléculas.

Depois de descoberta e optimizada a conjugação supracitada, diferentes modificações sintéticas foram feitas para conferir propriedades químicas específicas à molécula, mantendo a estrutura nuclear que permite a conjugação. Sintetizados vários compostos com diferentes propriedades, importantes estudos biológicos poderam ser feitos. Nomeadamente, o estudo que permitiu concluir que os iminoboronatos são estáveis o suficiente a um processo de internalização celular e que a conjugação de um fármaco citotóxico a uma biomolécula poderá aumentar a selectividade da internalização do fármaco. O estudo de reversibilidade intracelular do conjugado está neste momento a decorrer, através do desenvolvimento de uma sonda "ON-OFF" específica para este tipo de conjugações.

Ainda assim, no decorrer do trabalho, verificou-se que o sistema de conjugação tem uma reversibilidade deficiente, pelo que, foram iniciadas experiências (ainda em curso) para melhorar esta bioconjugação ao ponto de potenciar as suas aplicações biológicas.

Palavras-Chave: Modificação proteínas, lisina, ácidos borónicos, iminoboronatos, reversível

Chapter I

State of the Art

ABSTRACT

Proteins are biomolecules that carry out the majority of cell's functions, acting as their structural blocks, as catalysts and regulators of all processes concerning cell cycle or cellular metabolism. Not to mention, therapeutic peptides or proteins that show pivotal importance to some pathologies. However, since these molecules are unstable to several conditions, peptide and protein enhancing technologies are being developed in order to stabilize such biomolecules or to create more efficient delivery systems, either by conducting the protein itself or acting as a carrier for a given drug.

Selective modification of native proteins is one of the approaches to change a protein's properties without varying its function or natural structure. Usually, selective reactions that take place under naturing conditions are studied with the intention of modifying one amino acid, in the presence of several functional groups of a peptide/protein. Selective bioconjugation relies on amino acids' properties, whether for their reactivity, as much as for their occurrence in proteins or their accessible surface area.

In this chapter, a state of the art of chemical modification of proteins will be presented.

Chapter I.
State of the Art

I.1. Protein Modification

Proteins are biomolecules that are responsible for a wide variety of functions, namely as catalysts in cellular metabolism as regulators of cell cycle, differentiation, growth, division, motility, death, among others.[1] Moreover, proteins are vital members of a cell communication system that controls cellular activities and actions, called cell signalling. Considering the three most important classes of signalling molecules: hormones, neurotransmitters and cytokines, amino acid based biomolecules are the base for two of them. For example, peptide/protein hormones like TRH, vasopressin, insulin or growth hormone are invaluable molecules for a living organism, as well as cytokines that are a large group of small proteins that represent the core of all immune system.[2]

Many of those functions, crucial for the signalling network, are dependent on a tightly controlled system of *in vivo* chemical modifications of proteins, which are called post-translational modifications (PTMs). Examples of such reactions are phosphorylations, glycosylations, ubiquitinations, methylations or acetylations, each with its important role to perform a given function.[3] A clear example of the importance that these modifications have to a living organism are those operated on histones.

Histones are proteins found in eukaryotic cell nuclei that are wrapped around by the DNA helix creating cored structures named nucleosomes. These are, subsequently, folded into higher order structures called chromosomes. Despite the clear organization induced by these proteins to an otherwise disorderly DNA, they are also quite relevant in terms of gene regulation, and at this point is where PTMs play a substantial role. For example, histone acetylation and deacetylation are processes that occur at the ε-amine of the side chain of lysine residues within the *N*-terminal tail protruding from the histone core of the nucleosome. Acetylation removes the positive charge on the histones, thereby decreasing the interaction of the *N-termini* of histones with the negatively charged phosphate groups of DNA. As a consequence, the condensed chromatin is transformed into a more relaxed structure that is associated with greater levels of gene transcription.[4,5]

Other examples of important PTMs are those occurring on proline residues in the transcription factor HIFα, which are involved on oxygen-sensing mechanisms within cells and, also, the phosphorylation cascades that occur on different amino acids which are crucial for intracellular signalling.[6] All of which demonstrate the function modulation that is stressed by these “simple” peptide or protein modifications.

Nonetheless, to fully comprehend the biological role of PTMs and to determine precise structure-activity relationships, access to pure protein derivatives is crucial.[7] Natural protein production methods usually lead to complex PTM mixtures, making it difficult to determine which derivative is causing what effect. Therefore, chemical modification of proteins that allow the production of artificial post-translational modification mimics can be a useful tool to achieve pure mimics of complex PTMs or to create artificially modified proteins.[8]

Historically, chemical modification of proteins started with the chemistry of amino acids themselves and, only later, concerning the amino acid side chains of intact proteins. In the early 20th century, experiments were done based on empirical observations and as a result, determinant data was gathered, for instance, for the tanning industry or for toxicology. During this period a significant number of proteins were also crystallized. Before World War II, few analytical data of individual amino acid was available. After this historical landmark, identification of which amino acid was responsible for the biological activity of proteins (active sites) started to be the object of study. During the next twenty years, some techniques were studied that allowed a greater knowledge of these biomolecules: cation-exchanger amino acid analyser; radioactively labelled reagents; amino acid analysers or ion-exchange and gel exclusion chromatography. In 1962, the first selective modification was performed by Wofsey *et al.*, modifying a chymotrypsin's histidine that, consequently, eliminated its catalytic activity. During the 80's and 90's there was a large improvement of bioconjugation reagents and techniques, mainly enhanced by the evolution of mass spectrometry.[9]

More recently, with the production of recombinant proteins and their constant improvement, these biomolecules have become commercially interesting (estimated in $90+ billion U.S. market share in 2009).[10] This procedure allied with bacteria or yeast engineering to incorporate site-selective natural or non-natural amino acids on a protein sequence led to relevant developments on synthetic biology.[1]

Protein modifications enable the modulation of the biomolecule's properties and the tracking of its distribution *in vitro* or *in vivo*, using, for instance, fluorescence based techniques or affinity tagging. Moreover, therapeutic and signalling protein conjugates have become very promising constructs for studying pathologies, such as: HIV,[11,12] cancer,[11,13,14] malaria,[11,15] neurodegenerative diseases like Alzheimer's disease,[15] acromegaly[13] or *Angina Pectoris.*[12] The most ambitious goal in the field of protein modification is to create conjugates and biomarkers relevant for the development of new therapeutic molecules or systems that lead to a more effective and targeted therapy.[16]

I.2. Site-Selective Modification

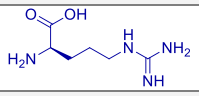
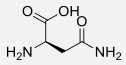
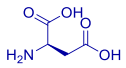
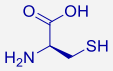
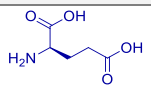
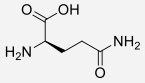
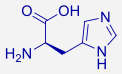
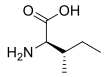
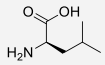
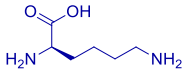
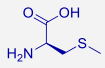
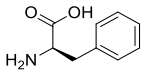
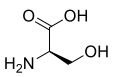
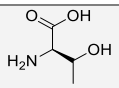
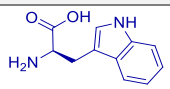
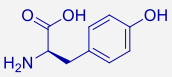
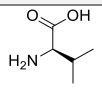
Selective chemical modification of proteins is a very wide area of research that can be divided into three different groups, each facing different limitations: modification of natural amino acid side chains, modification of terminal residues or modifications of non-natural amino acids. Each of these groups will be discussed in detail. When considering a site-selective modification, the functionalization of a single residue in the presence of multiple unprotected functionalities is a remarkable chemical challenge. Particularly, in order to have broad applicability, these methodologies need to operate in conditions that prevent unfolding of the protein, namely these reactions need to be fast at low temperature and at near neutral pH. Furhermore, proteins are only available in low concentrations, and therefore the modification reactions have to occur at high dilution. All these requirements limit the choices of chemical reactions that can be used for this purpose.[11] Nevertheless, several examples have already been studied and will be presented below.

I.2.1. Modification of Natural Amino Acids

With the development of techniques such as genetic engineering, a new possibility for modification of proteins has emerged. Site-selective modification of proteins through reaction with amino acids incorporated at specific sites of a protein's sequence makes it possible to overcome a number of limitations that conjugation to non-recombinant proteins usually face. These limitations are often related with the latent properties of the amino acids, whether for their natural occurrence and accessible surface, as for their chemical reactivity (Table **1**).

Nine of the canonical amino acids can be referred as chemically active, and, thus suitable for natural amino acids modification (highlighted in Table **1**), namely, acidic residues (aspartic acid and glutamic acid), aromatic amino acids (tyrosine and tryptophan), sulfur containing amino acids (methionine and cysteine) and nitrogen containing side chains (histidine, lysine or arginine, as well as *N*-terminal function). Of all the properties mentioned in the table, reactivity of the amino acid side chain is the most efficiently explored. Exactly for this reason, cysteine and lysine, having the best nucleophiles in their side chains, are the residues most commonly modified.[1,10] Less often, carboxylate or hydroxyl functionalities can also be targeted, as being nucleophiles, though a lack of selectivity is often observed due to the frequent presence of functional groups with similar characteristics.[17] For this reason, modification of cysteines and lysines will next be discussed in more detail, while the functionalization of other groups will be briefly summarized.

Table **1** – Amino acids' properties[a]

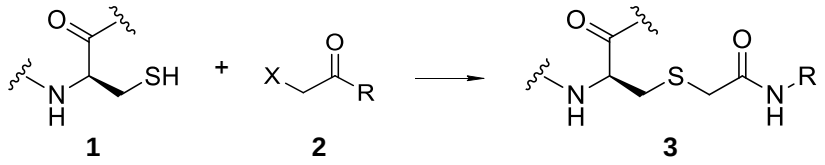
Amino Acid		pK_a of ionizing side chain	Occurrence in proteins (%)	Buried residues (%)	Accessible surface area (A^2)
Alanine (Ala, A)		-	7.5	38 (12)	67
Arginine (Arg, R)		12.5 (>12)	5.2	0	196
Asparagine (Asn, N)		-	4.6	10 (2)	113
Aspartic Acid (Asp, D)		3.9 (4.4 – 4.6)	5.2	14.5 (3)	106
Cysteine (Cys, C)		8.3 (8.5 – 8.8)	1.8	47 (3)	104
Glutamic Acid (Glu, E)		4.3 (4.4 – 4.6)	4.1	6.3 (2.2)	144
Glutamine (Gln, Q)		-	6.3	20 (2)	138
Glycine (Gly, G)		-	7.1	37 (10)	-
Histidine (His, H)		6.0 (6.5 – 7.0)	2.2	19 (1.2)	151
Isoleucine (Ile, I)		-	5.5	65 (12)	140
Leucine (Leu, L)		-	9.1	41 (10)	137
Lysine (Lys, K)		10.8 (10.0 – 10.2)	5.8	4.2 (0.1)	167
Methionine (Met, M)		-	2.8	50 (2)	160
Phenylalanine (Phe, F)		-	3.9	48 (5)	175
Proline (Pro, P)		-	5.1	24 (3)	105
Serine (Ser, S)		-	7.4	24 (8)	80
Threonine (Thr, T)		-	6.0	25 (5.5)	102
Tryptophan (Trp, W)		-	1.3	23 (1.5)	217
Tyrosine (Tyr, Y)		10.9 (9.6 – 10.0)	3.3	13 (2.2)	187
Valine (Val, V)		-	6.5	56 (15)	117

[a] For more detail, see reference [18]

I.2.1.1. Cysteine

Cysteine has a sulfhydryl group on its side chain, which makes it the strongest natural nucleophile of a protein. Therefore, this functionality is the easiest to manipulate selectively with chemical reagents, even at neutral pH.[13] Another important characteristic of cysteines that allows the site-selective functionalization of proteins is its scarce occurrence in proteins (1.8%)[19,20] conferring cysteine modification a very high specificity but a limited number of applications without resorting to genetic manipulation of proteins or reducing agents (used to disrupt natural occurring disulfide bridges).

The first reaction that modified the side chain of cysteine was an alkylation using an appropriate electrophile, which goes back to 1935. *Goddard and Michaelis* used iodoacetamides to alkylate cysteines of queratine.[21] The reaction had its flaws (crossed reactivity with other nucleophiles, such as lysine), but it was an important starting point in research of different electrophiles suitable for this purpose. From these, other *α*-halocarbonyls emerged (**2**, Scheme **1**), such as chloroacetamide or *α*-bromoacetophenone derivatives, which were more selective and permitted the alkylation of the active center of papain, transforming this cysteine protease into an inactive enzyme.[22]

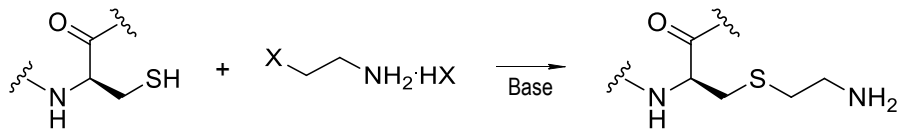


1 **2** **3**

Scheme **1** – Cysteine modification – *α*-Halocarbonyls, X = Cl, Br or I and R = NHR' or Ar

Nucleophilic substitution for protein modification continued to be evaluated over the years, leading to other interesting studies, such as the production of lysine mimics by aminoethylation (Scheme **2**), or the introduction of chemical haloaryl functions (Scheme **3**) or the generation of glycoproteins using halo-glycosyl donors.[23]

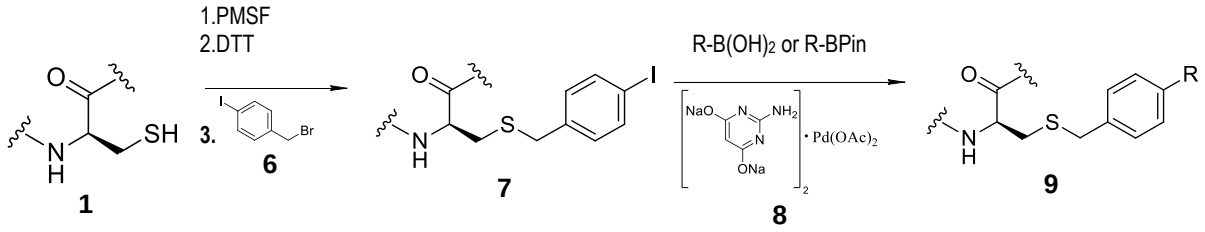
The lysine mimetics were obtained *via* reaction of the cysteine thiol group with aziridines, prepared *in situ* with *β*-haloethylamines, **4**, producing a thioether with a terminal primary amine (**5,** Scheme **2**).[11]



1 **4** **5**

Scheme **2** – Cysteine modification – *β*-Haloethylamines, X = Cl or Br

Later, the cysteine thiol group was modified to incorporate a halogenated aromatic moiety that was subsequently used to perform a *Suzuki-Miyaura* cross-coupling reaction (Scheme **3**). By using 1-bromomethyl-4-iodobenzene, **6**, for the nucleophilic substitution and palladium (0) complex, **8**, to catalyze the cross-coupling, Davis *et al.* modified a recombinant protein under mild conditions (phosphate buffer, pH 8.0, at 37 ºC) leading to the formation of a glycoprotein, among other examples.[24]

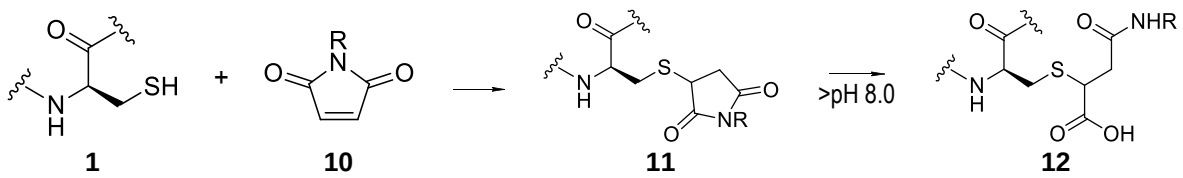


Scheme **3** – Cysteine modification leading to cross-coupling *Suzuki-Miyaura*

Another example of alkylating agents that have been largely used for cysteine modification are Michael acceptors like acrolein, vinyl sulfones, alkynoic amides and maleimides.[11,13,19,25–28]

Acrolein being one of the strongest electrophiles among all *α,β*-unsaturated aldehydes was shown to be unselective, exhibiting cross-reactivity with histidine and lysine. Nevertheless, it was used to promote structural modifications of soy protein.[29]

Over the years, maleimides (**10**) have been the most used scaffold to label cysteine residues. These Michael acceptors can react rapidly and selectively with thiols at slightly acidic to neutral pH (6.5-7.5), generating a thioether bond, **11** (Scheme **4**). Above pH 8.0, maleimides are hydrolytically labile, forming nonreactive maleamic acids (**12**, Scheme **4**) and present cross-reactivity with amino groups. The construct hydrolysis is also dependent on the chemical nature of the maleimide *N*-substituent.[30] Furthermore, recent studies on the stability of maleimide-conjugates in human plasma and *in vivo* assays, highlight a cross-reactivity of these conjugates with reactive thiols from albumin, free cysteine or glutathione, if the maleimide conjugation site is highly solvent accessible or if the hydrolysis is promoted by a positively charged environment at partially accessible cysteines.[31]



Scheme **4** – Cysteine modification – Maleimides

This Michael addition is one of the most studied and understood reactions for bioconjugation and has been used in the construction of several bioconjugates.

The extensive use of this bioconjugation scaffolds resulted in a detailed knowledge of the properties of this molecule in terms of conjugation and stability of the constructs and now, maleimides are often used as reference when designing new reagents for protein modification.[32–36] Moreover, maleimides are currently commercially available to apply different biological applications.

Due to the large number of applications in which maleimides have been used, an organization based on the recurrence of the application was made. The three most important groups of applications either use maleimides to develop potential diagnostic tools and therapeutic agents, or to grant certain biological properties into biomolecules or as one of the counterparts of heterobifunctional linkers.

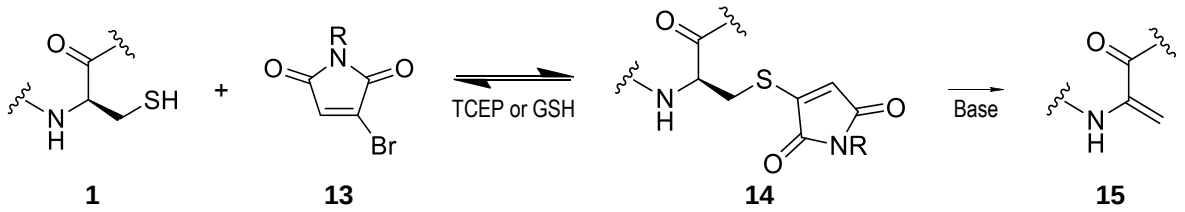
Several strategies have been pursued for developing novel therapeutic agents based on maleimide conjugation, though two are particularly important. PEGylated maleimides have been used to improve the pharmacokinetics properties of biomolecules such as therapeutic peptides or proteins. In another field, maleimides have been extensively used to conjugate drugs to biomolecules that are selectively recognized by a specific target. The synthesis of antibody-drug conjugates (ADCs) is one of the most important examples. Drugs combined to an antibody by means of maleimide conjugation are one of the most recurring methods and led to the development of an Food and Drug Administration (FDA) approved ADC, named brentuximab vedotin (Adcetris®).[37–41]

As examples of maleimides that have been conjugated to proteins to grant some originally absent biological properties, artificial glycosylation by means of glycosylmaleimides can be mentioned or even the incorporation of biotin-maleimides in order to have a recognizable agent for surface immobilization.[16,42–44]

Lastly, another common application that uses maleimide conjugation is based on the development of bifunctional linkers that attach molecules through different conjugation systems. There are two main strategies with these maleimide heterobifunctional linkers: one that introduces bioorthogonal groups (such as azides, alkynes or olefins) on the opposite end of maleimides and another that introduces amine reactive groups (*e.g. N*-hydroxysuccinimide (NHS) esters) for the same purpose.[45–49]

As an alternative for maleimides, bromomaleimides (**13**) were described in 2009 as reagents for selective and reversible modification of cysteine (Scheme **5**). This reversibility feature was introduced to an otherwise irreversible reaction, which could be extremely useful for temporary modifications. According to the authors, this reversible modification of cysteine could be used for transitory blockage of an active

cysteine residue, cysteine conversion into dehydroalanine (Dha, **15**, Scheme **5**), protein purification, quantitative proteomic analysis, probing of binding sites, possibility of structural studies or even drug delivery.



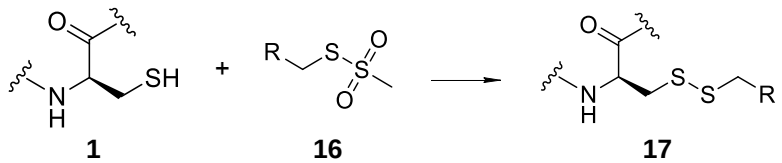
1 **13** **14** **15**

Scheme **5** – Cysteine Modification – Monobromomaleimides

A year later, Caddick, Baker *et al.* reported a system to reversibly modify a cysteine residue incorporated in a mutant protein, enabling a controlled separation between the conjugation molecule and labeled protein. In the same study, bromomaleimides were proven as plausible molecules to create pro-drugs, since they, presumably, present a controlled release in the cell's interior, when in the presence of higher concentrations of glutathione.[13]

Exploring the structural versatility of bromo and thiomaleimides,[50–52] these molecules have been used in artificial glyscosylation, biotinylation, PEGyaltion, development of fluorescent bromomaleimides and, more recently, ADC's production.[51,53–58]

Oxidation of cysteine is an additional method that can be used to modify this residue in a protein, in order to form disulfide bridges between the thiol-side chain and alquilated reagents of methanethiosulfonate (MTS, **16**, Scheme **6**) or other thiol-disulfide exchange reagents, such as pyridyl disulfides or 5-thio-2-nitrobenzoic acid (TNB)-thiol-activated species.[11,30]



1 **16** **17**

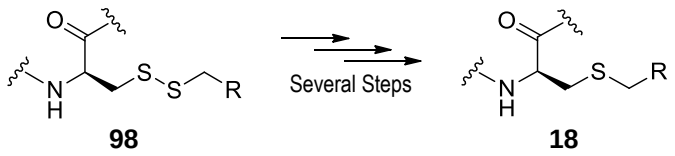
Scheme **6** – Cysteine modification – Alquilated reagents of methanethiolsulfonate

Through derivatization with MTS, Jones *et al.* varied the specificity of two pockets (S_1 e S_1') of subtilisin in *Bacillus lentus* and Davis *et al.*, using this approach was able to modify important substrates that were

shown to be sensitive systems to detect inflammatory illnesses of mammal brains, like Malaria or Alzheimer's disease.[15,59]

Remarkably, using another thiol-disulfide exchange reagent (a TNB-thiol-activated species), Neri *et al.* recently applied this approach for the development of linkerless ADCs where a thio-drug was directly conjugated with antibody fragments. These ADCs, in the presence of reducing agents such as glutathione, unconjugate without any trace of linker attached to either counterpart, thus developing a traceless conjugation method.[60–62]

Since the disulfide bridge formed during the oxidation of cysteine is sensitive to reducing environments and this can be a limitation for some applications, it was important to find a procedure to reduce this linkage to more stable thioethers (Scheme **7**). With this purpose, Davis *et al.* developed such procedure using phosphines and applied it in the reduction of an oxidized form of cysteine in a mutant protein (SBL-S156C) at basic conditions (phosphate buffer pH 9.5).[63]



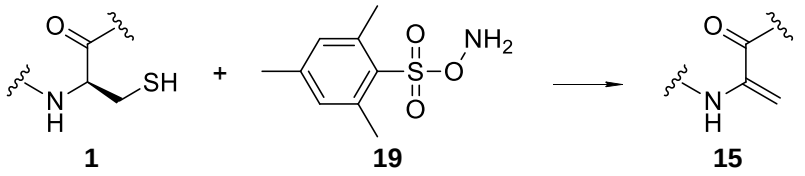
98 **18**

Scheme **7** – Cysteine modification – Reduction of previously forms of oxidized cysteines

Apart from the already discussed additions, substitutions, oxidations and reductions of cysteine and derivatives, there is a strategy that converts the most nucleophilic residue among all natural amino acids (Cys) into an electrophilic species (Dha). Since proteins do not have natural occurring electrophiles this new formed species becomes a valuable synthetic precursor to a wide collection of protein modifications.[64] There are different methods to install Dha on the protein surface based on the elimination of cysteine, which include the reduction-elimination of cysteine-disulfides, the base-mediated elimination of cysteine-disulfides, the oxidative-elimination of cysteine and the bis-alkylation-elimination of cysteine.[65]

The first two strategies are based on the previously described method of oxidation of cysteines to generate disulfides, which can then be reacted with phosphines (regioselective reduction with tris(dimethylamino)phosphine (HMPT)) or bases (*e.g.* 1,8-Diazabicycloundec-7-ene (DBU)) to further convert these residues.

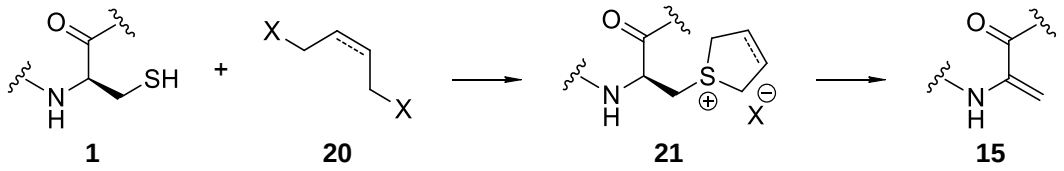
The oxidative-elimination of cysteine to obtain Dha uses *O*-mesitylenesulfonylhydroxylamine (MSH, **19**) as the oxidizing agent in a phosphate buffer pH 8.0, during 20 min, at 4 ºC (Scheme **8**).[66] Later, some side-reactions of this method were presented with other nucleophilic amino acids (Asp, Glu, Met, Lys, and His) and with the *N*-terminal amino group.[65]



1 **19** **15**

Scheme **8** – Cysteine modification – Dehydroalanine generation (1)

In order to avoid this cross-reactivity, the same authors tested a potentially more selective transformation: the bis-alkylation–elimination of cysteine to dehydroalanine. Several reagents were tested to achieve a fast formation of the 5-membered ring sulfonium intermediate and subsequent elimination to generate Dha. Different water-soluble reagents with the required 1,4-dihalobutane moiety were evaluated in order to grasp the optimal intended modification (Scheme **9**), which led to the modification of SBL-S156C with bisamide of the 1,4-dibromobutane core, at pH 8.0. However, this procedure requires incubation for one h at 37 ºC in order to induce cyclization and elimination.



1 **20** **21** **15**

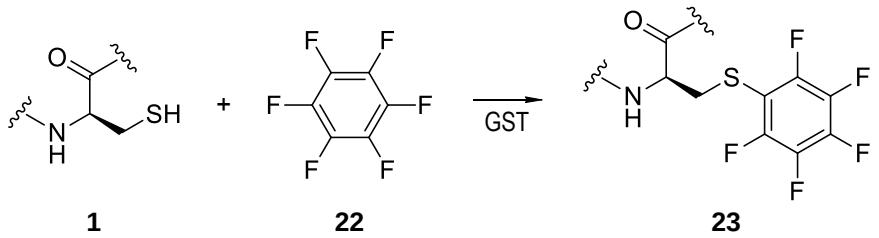
Scheme **9** – Cysteine modification – Dehydroalanine generation (2)

Later, Caddick *et al.* reported an unexpectedly stable cyclic sulfonium intermediate, which was explored as a different strategy to modify cysteines, enabling a site-selective dual labelling of a mutant green fluorescent protein (GFP) using the same cysteine modifying reagent (2,5-dibromohexanediamide).[67,68] The ring opening of the stable cyclic sulfonium intermediate was selectively obtained by treatment of the construct with sodium azide (which was then reacted with a commercially available strained alkyne dye) and Dha was reacted with a thiol dummy in order to show selective reactivity for both electrophiles.[68]

Regarding other methods to modify cysteine, it is worth mentioning a click process based in addition reactions of thiols to alkenes or alkynes, designated as thiol-ene coupling (TEC) and thiol-yne coupling (TYC). These processes rely in a radical mechanism, in order to give anti-Markovnikov-type thioether.[69] Dondoni and Davis developed a system based in this free-radical hydrothiolation of alkynes to give a dithioether derivative with exclusive 1,2-addition, showing as applications artificial glycosilation and fluorescent labeling of proteins.[70]

A series of new reagents for selective modification of cysteine residues were recently reported and they present interesting characteristics that show promising applications. These reagents include perfluoroaromatic molecules, 2-cyanobenzothiazole, methylsulfonylbenzothiazole, phenyloxadiazole and allenamides. [32,71–80]

Pentelute *et al.,* in 2013, reported the discovery of a nucleophilic aromatic substitution (S_NAr) between perfluoroarenes and cysteine residues from unprotected peptides, at room temperature (Scheme **10**). These authors describe their conjugation system as a method to access rigid perfluoroaromatic staples that fulfils several requirements of "click" chemistry.[79] Nevertheless, the insolubility and low reactivity of such reagents in aqueous media led them to turn their attention to an enzymatic "click" ligation promoted by glutathione S-transferase (GST) which is responsible for cellular xenobiotics detoxification *in vivo*. This enzyme recognizes the glutathione moiety and performs a site-specific modification of its cysteine residue with an activated perfluoroaryl electrophile in the presence of other unprotected cysteine residues, as well as other reactive functional groups.[72]



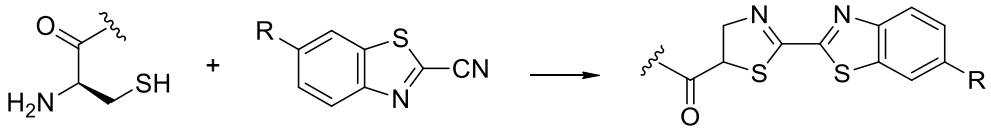
1 **22** **23**

Scheme **10** – Cysteine modification – Perfluoroaromatic Molecules

In 2009, Rao *et al.* described the modification of an *N*-terminal cysteine residue with 2-cyanobenzothiazole (**25**, CBT) derivatives (Scheme **11**) by transposition from a reaction used in the last step of the synthesis of D-luciferin (a common substrate for firefly luciferase) that occurs smoothly in aqueous media. During this first assay, the authors synthesized three CBT derivatives, two fluorescent

ones and a biotin-derivative, which were used to show that this condensation proceeds with a high degree of specificity and efficiency, at physiological conditions, *in vitro* and on cell surfaces.[77]

A year later, the same authors described an interesting study that allowed direct imaging of the condensation products generated by glutathione reduction and proteolytic hydrolysis inside cells revealing their different subcellular locations, demonstrating the feasibility of a controlled and localized reaction in living cells. This intracellular condensation process enabled the imaging of the proteolytic activity of furin which can be a good indication for *in vivo* molecular imaging of cancer and selective drug delivery to tumor cells that often overexpressed proteases like furin and cathepsins.[80] In 2012, Rao, Chin *et al.* presented an efficient method for site-specific ^{18}F-labeling of cysteine-bearing peptides and proteins, by developing ^{18}F-CBT which was used to show good *in vivo* tumor targeting efficiency for positron emission tomography (PET) imaging. However, the increased accumulation of their conjugate in livers showed that this structure needs to be optimized in order to increase hydrophilicity.[73]

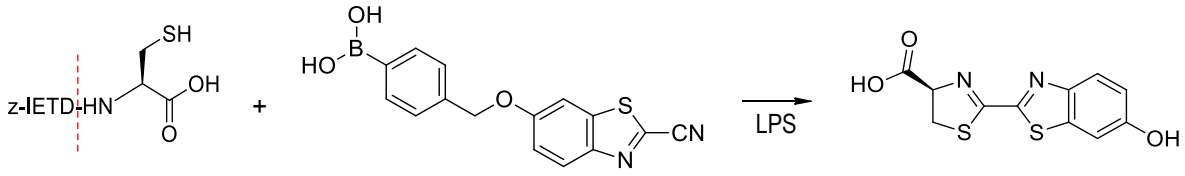


24 **25** **26**

Scheme **11** – *N*-terminal cysteine modification – 2-Cyanobenzothiazole

Following this work, Chin *et al. were* able to genetically encode an unnatural amino acid featuring 1,2-aminothiols (as mimetics of *N*-terminal cysteines) in the side chain, thus facilitating this bioorthogonal condensation[75] and Lin *et al. were* able to promote such condensation to apply a novel site-selective protein immobilization method.[74]

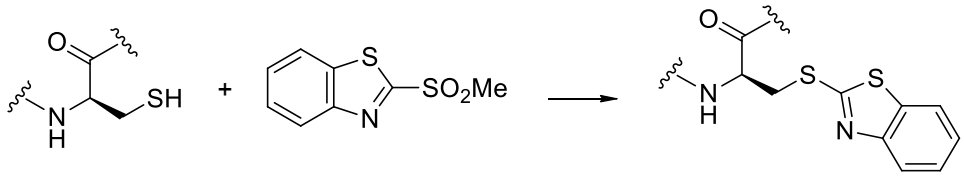
In 2013, Chang *et al.* developed an ingenious strategy for dual analyte detection in living systems that was mostly based on this condensation. Peroxy Caged Luciferin-2 (**28**) was developed, bearing a H_2O_2-responsive boronic acid probe that releases CBT-6-OH upon reacting with reactive oxygen species, which in turn reacted with a *N*-terminal $_D$-cysteine released from a peptide-based probe (**27**) in the presence of active caspase 8, to generate firefly luciferin *in situ* (Scheme **12**). In this case, the bioluminescent signal from luceferin represents the presence of reactive oxygen oxygen species and caspase 8 activity. This shrewd method offers a potentially powerful tool for studying simultaneous oxidative stress and inflammation processes in living animals during injury, aging and other pathological abnormalities.[76,81]



27 **28** **29**

Scheme **12** – Dual-Analyte Luciferin Imaging; LPS - Lipopolysaccharides

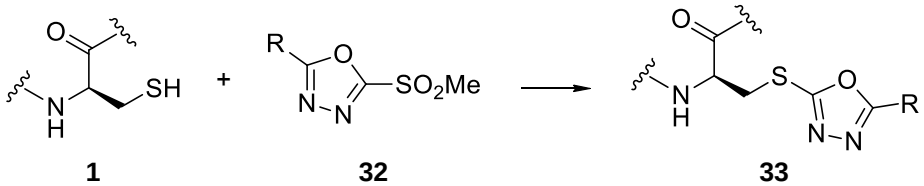
In 2012, Xian *et al.* described the use of similar probes as electrophiles in a selective nucleophilic substitution. After attempting different leaving groups at the position two of the benzothiazole, the authors described methylsulfonyl benzothiazole reagent (**30**) as a selective protein thiol blocking agent (Scheme **13**). This study evaluated the selectivity when using this reagent at different pHs and against different amino acids with different nucleophilic side chains (namely, tyrosine, serine, lysine, histidine, methionine and tryptophan), revealing that the substitution occurs faster in basic pHs, though, at the same time, it promotes cross-reactivity with amines.[82]



1 **30** **31**

Scheme **13** – Cysteine modification – Methylsulfonyl benzothiazole

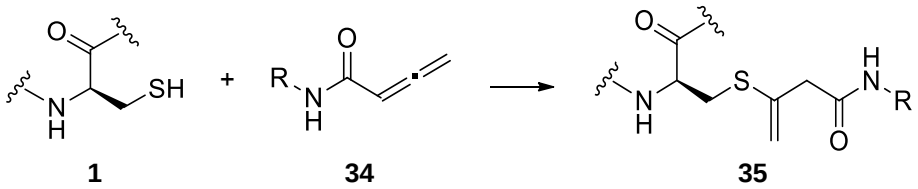
Inspired by this work, a year later, Barbas III *et al*. synthesized a series of methylsulfonyl-functionalized monocyclic compounds to be used as selective thiol alkylating reagents. This study revelead that phenyloxadiazole (**32**) reacts with cysteine-protected amino acids to afford quantitatively the conjugate in less than 5 minutes (Scheme **14**). The authors also demonstrated that such scaffolds are amenable to synthetic manipulation allowing the site-selective introduction of fluorophores, azides and poly(ethylene)glycol chains at the surface of proteins. The phenyloxadiazole diapoles were also shown to produce more stable constructs than the ones obtained with maleimides.[32]



1 **32** **33**

Scheme **14** – Cysteine modification – Phenyloxadiazole

Finally, very recently, allenamides (**34**) have been described as novel orthogonal handles for selective modification of cysteine in fully unprotected peptides and proteins (Scheme **15**). These reagents show great promise due to a combination of factors, such as the fast kinetics of *C*-substituted allenamides towards thiols, its unreactivity towards amine and hydroxyl nucleophiles and their stability in aqueous media. This conjugation method also permit the synthesis of stable and irreversible conjugates with interesting characteristics.[78]



1 **34** **35**

Scheme **15** – Cysteine modification – Allenamides

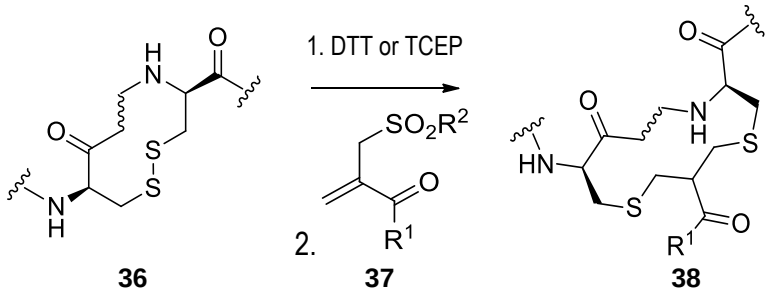
These novel modifications of cysteine residues are expected to produce valuable applications in a near future and shouldn't be disregarded while thinking about alternatives to more classic methods of conjugation, such as the one with maleimides.

To close the topic of cysteine modification it is required to mention the disulfide bridging reduction-alkylation strategy, since a vast portion of relevant natural proteins do not have free unpaired cysteines, available for modification. Usually, proteins present an even number of cysteines that pair up to form disulfides, which are considered to be crucial for tertiary structure and biological activity, thus, seen as unsuitable targets for chemical modification. However, since usually one has to use recombinant engineering to introduce a free cysteine for further modification, a strategy that reduces selectively naturing occurring disulfides using mild reducing conditions presents a valuable alternative for selective and efficient addition chemistry of thiols.[83,84]

By separating disulfide bridges according to their solvent accessibility, Shaunak, Brocchini *et al.* showed that it was possible to use dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) to reduce a disulfide bridge to its two free cysteine sulfur atoms, maintaining the protein's tertiary structure, as the buried disulfides remained intact. This would only be possible without using denaturants in order to ensure that inaccessible disulfides are not reduced. After reduction, a bis-alkylation conjugation is performed to reconnect the two cysteine sulfur atoms *via* a three-carbon bridge or a two-carbon bridge.[83]

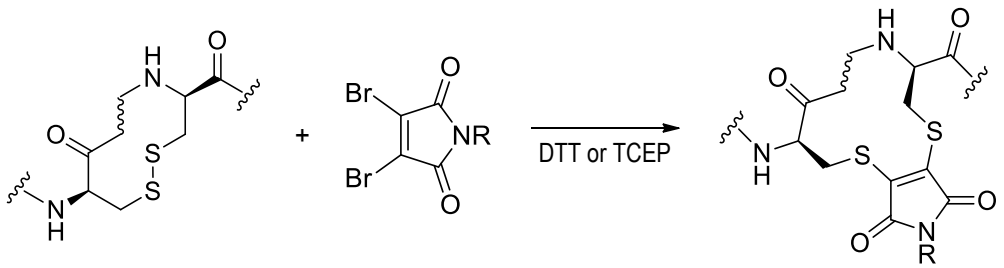
In 2005, Alley *et al.*, members of Seattle Genetics, studied the possibility of achieving precise control over the location of the conjugation in the preparation of homogenous ADCs. This was obtained by chemical reduction and oxidation methods that lead to preferential cleavage of particular monoclonal antibody interchain disulfides and subsequent alkylation with maleimides.[85] This pioneer study set the basis for selective bioconjugation on disulfide rebridging using chemical reagents specially designed for this purpose.

In 2006, Shaunak, Brocchini *et al.* described one of those rebridging disulfide procedures using cross-functionalized PEG monosulfone (**37**) to generate a three-carbon bridge between two sulfur atoms that were previously reduced from a disulfide bridge (Scheme **16**). The α,β-unsaturated ketone present in compound **37** is require to initiate the sequence of addition-elimination reactions, which starts from the 1,4-addition of the thiol leading to an elimination of sulfinic acid, generating another conjugated double bond for the second thiol addition.[84] Several PEGylation systems were developed by this group in the following years for different applications, leading to the production of a stable ADC.[35,83,86–89]



Scheme **16** – Disulfide bridge modification – Monosulfone

In 2010, Caddick, Baker *et al.* developed a different rebridging disulfide procedure using dibromomaleimides (**39**) to generate a two-carbon bridge without the requirement of an additional asymmetric carbon (Scheme **17**). The authors started from synthesizing *N*-fluoresceindibromomaleimides to conjugate somatostatin's disulfide bridge in order to demonstrate this idea, then the authors tried to optimize this conjugation by changing bromo for other good leaving groups, like chloro, iodo, dimercaptoethanol and dithiophenol substituents.[51,53] Dibromo and dithiomaleimides proved to be the most efficient reagents for this procedure.[54,55,90]



36 **39** **40**

Scheme **17** – Disulfide bridge modification – Dibromomaleimides

In conclusion, several reagents have been used to selectively modify cysteine residues, since this amino acid has singular characteristics ideal for chemical modification. From simple nucleophilic substitutions and conjugate additions, through thiol-dissulfide exchange reagents and their reduction, as well as dehydroalanine generation and transition-metal-catalysed C-C bond-forming reactions, not to mention the novel chemical reagents for selective modification of cysteine and the disulfide bridge reduction-alkylation procedure, all show promising results to obtain even more selective and homogenous protein and peptide conjugates. Probably, there are still new cysteine modification agents yet to emerge as the demand for specificity of conjugation sites becomes firmer over the time. This requirement is even amplified when therapeutic strategies and fundamental biological studies are envisioned.

I.2.1.2. Lysine

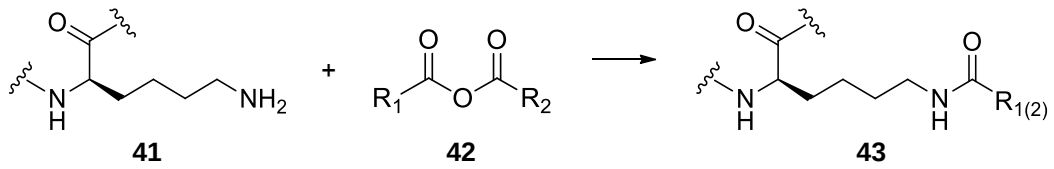
Another natural amino acid (NAA) that is largely used for protein modification due to its natural characteristics is lysine. This is an amino acid with a primary amine on its side chain, making lysine the second best nucleophile of a protein, apart from cysteine.[28] It has been reported that this *ε*-amine can be distinguished from other *α*-amines on a protein's surface through its pKa (pKa of 9.3–9.5 for lysine versus pKa of 7.6–8.0 for *α*-amines), but depending upon the reaction's selectivity, cross-reactions can occur with other nucleophilic residues, such as the imidazol group of histidine, or the hydroxyl function of serine, threonine or tyrosine, and the thiol group from cysteine, as well as, the most common interferent, the *α*-amine group of *N*-terminal functions.[12] In addition to this, lysines are also often positioned on the surface, making it available to be simply derivatize.[30] The modification of lysines typically generates heterogenous constructs, which depending on the application, may be considered as a limitation.

Although lysine is a considerably more abundant amino acid than cysteine, this residue is a common target for modification due to its nucleophilicity, surface exposure and abundance of methods to selectively aim primary amines.[91] The most common reagents to modify these amines proceed *via* the

acylation or alkylation of the ε-amine group, yielding stable amides or secondary amines, respectively. Examples of such reagents are: acyl azides, activated esters (NHS and fluorophhenyl esters), carbodiimides and anhydrides that form amide bonds and aldehydes and glyoxals (with subsequent reduction), epoxides and oxiranes, arylating agents that yield secondary amines. Other groups of reagents to obtain different or the same linkages are: iso(thio)cyanates, sulfonyl chlorides, carbonates, imidoesters, hydroxymethyl phosphine derivates, *O*-alkylisoureas, diketene,[92] ethyl squaramyl group,[93] imidazole-1-sulfonyl azide,[94] aldehyde terpenoids (such as (*E*)-3-methoxycarbonyl-2,4,6-trienal) and reductive alkylation with iridium.[28,30,95]

Despite having this much modifications reported, some are more recurrent then others. For that reason, anhydrides, isocyanates, activated esters and different aldehydes will be discussed in further detail, together with other very promising studies for future application. It is of note that most of these reagents can additionally modify the *N*-termini of proteins, though since usual heterogeneity is already obtained (due to a high abundance of lysine), this modification does not add crucial relevance.[91]

Anhydrides (**42**) were one of the first lysine derivatization group of reagents to be used. These reagents perform an acylating reaction with amines (preferentially at pH ~8 or above, Scheme **18**), but present cross-reactivity with residues bearing hydroxyl functions.[96]



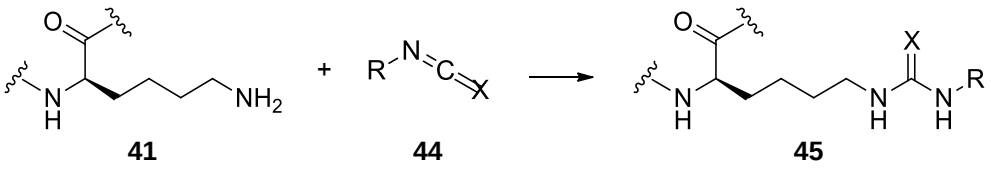
41 **42** **43**

Scheme **18** – Lysine modification – Anhydrides

In the late sixties, several anhydrides were described as reversible blocking agents of amino groups, starting from maleic and citraconic, moving to acetic, trifluoroacetic, succinic and tetrafluorosuccinic anhydrides.[97] Other examples could be named to demonstrate the vast applicability of these molecules,[98] but it is far more pertinent to give an impression of their evolution. This group of reagents started by using symmetric anhydrides (where R_1=R_2, **42**, Scheme **18**), as the ones aforementioned, and evolved for more complex structures of mixed anhydrides (where R_1≠R_2, **42**, Scheme **18**), in which mixed carbonic anhydrides and cyclic anhydrides are the representative sets.[99] However, this does not mean that symmetric anhydrides are not used currently, since, even to this day, a common method for protection of amino groups in solid phase peptide synthesis (SPPS) is to use an excess of a symmetric anhydride (acetic anhydride) to avoid side reactions.[100,101] As for all well-known reagents for protein modification,

these agents also present a vast number of applications already tested. Freeman *et al.* presented a study of protein modification with different anhydrides that led to changes in the crystals' porosity, which in turn made significant differences in terms of solubility or crystals shape.[98] Other applications for anhydrides include the study of changes in enzymatic activities, PEGylation of biomolecules or the introduction of chelating agents to an antibody.[37,38,102–106]

Alongside with anhydrides, isocyanates (X= O, **44**, Scheme **19**) and isothiocyanates (ITC, X= S, **44**, Scheme **19**) were also reported as reagents that rapidly modify primary amines, forming an urea or thiourea bond, respectively (Scheme **19**). Despite differences in cross-reactivity, both classes of reagents are susceptible to react with other nucleophiles of a protein. However, both of these cyanates are mainly used to modify primary amines since that is the only reaction that leads to stable products.[30] This reaction has an optimal pH higher than the physiological pH, requiring a more basic medium to react preferentially with amines (pH 9.0 to 9.5), which makes them unsuitable to modify alkaline-sensitive proteins.[28]

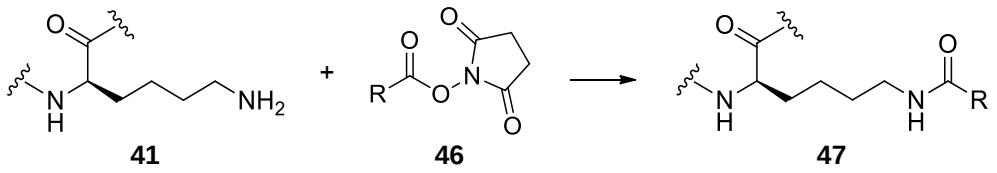


Scheme **19** – Lysine modification – Isocyanates, X=O or S

Isocyanates and isothiocyanates present as a major difference the different reactivity towards hydroxyl and thiol groups. Isocyanates are reagents that modify hydroxyl bearing amino acids, forming urethane linkages, and this has been used to perform the PEGylation of several biomolecules.[37] ITCs are known to react with cysteine forming dithiocarbamates, which are unstable at physiological conditions. Nevertheless, they have been reported as modifying agents of Cys347 of *α*-tubulin (inhibiting cell growth) and Cys79 of a binding pocket from a bacterial quorum-sensing regulator, LasR.[107,108]

Both cyanates mentioned are known to form many adducts with nucleophilic molecules and their toxicity towards living organisms is well known. A large number of studies were performed in order to justify isocyanates' toxicity.[107,109–111] Despite this, several applications for bioconjugation with these reagents have been disclosed in the literature. These include artificial glycosylation,[42] modification of virus' capsids,[112] antibody conjugation to introduce chelating agents,[113,114] incorporation of radioactive labeling agent (^{18}F) to lysine-containing proteins[115] and last, the most common application for these molecules, protein (e.g. antibodies) modification to introduce high quantum yields fluorochromes, such as the commercially available fluorescein-5-isothiocyanate (FITC) or rhodamine-B-isothiocyanate (RITC). These probes are known to maximize the detection sensitivity in binding assay or cell imaging.[116,117]

Later, in the mid seventies, activated esters were reported as acylating agents, and they rapidly became one of the most used molecules for protein bioconjugation. Among these activated esters, *N*-hydroxysuccinimide (NHS) esters (**46**) are the most explored acylating compounds and they were first described as homobifunctional crosslinkers.[30] Such reagents have an improved reaction specificity and a roughly 1000-fold lower required concentration when compared with a symmetric anhydride.[118] These reactive esters form an amide bond in the presence of unprotonated amines, using an aqueous environment of pH 8.5 ± 0.5 (Scheme **20**).[119,120] At lower pHs, *N*-terminal function and tyrosine can react preferably.[119] Over the years, several studies of chemical cross-linking have been reported using this functionality and they highlight cross-reactivity of NHS esters with *N*-termini, tyrosines, serines and threonines, which can be enhanced by basic amino acids (such as histidine) in their proximity.[119,121] Moreover, from the improvement of these reagents and the bioconjugation system, efficient heterobifunctional cross-linkers were developed and sulfonated derivatives of NHS esters were synthesized, enhancing the hydrosolubility of these species.[28,122–124]



41 **46** **47**

Scheme **20** – Lysine modification – NHS esters

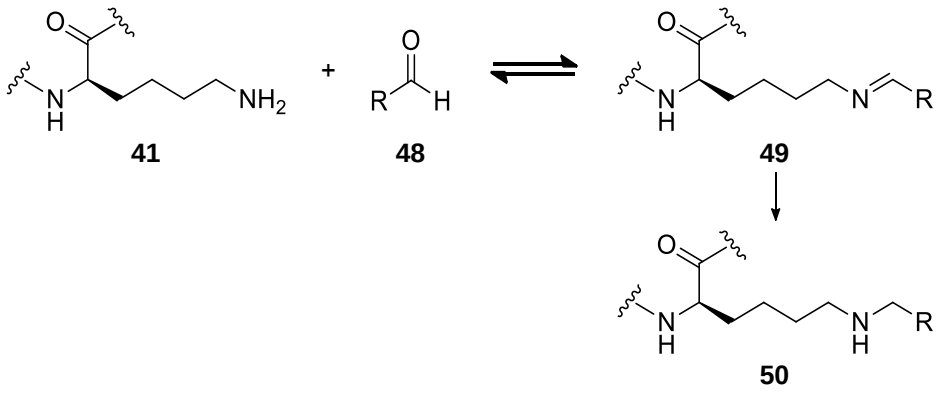
As the most used cysteine's reactive agent (maleimide), also NHS esters present numerous bioconjugation applications. The readly synthesis of NHS esters, a functionality that is commonly prepared by reacting a given acid with *N*-hydrosuccinimide and carbodiimide, also explains the wide variety of applications reported using these reagents. These applications include cell imaging and fluorescence studies,[125,126] constituent in numerous heterobifunctional linkers with maleimides,[127,128] hydrazines,[129,130] alkynes and alkenes (terminal and cyclic),[131–134] cyclic nitrone[135] and other bioorthogonal functions,[136] as well as, a reference molecule for different comparative studies with amine modifying reagents (ketenes, glutaldehydes)[137,138] and thiol modifying agents (dibromomaleimides).[55]

From all applications of NHS esters, available in the literature, probably, the ones that permit a direct intervention in a living system can be considered as the most interesting ones, whether as a diagnostic tool or a therapeutic agent.

As a representative example some FDA/EMA approved drugs with different indications based on NHS methodology can be mentioned, such as: two ADCs (Mylotarg® and Kadcyla®) and two PEGylated cytokynes (PegIntron® and Pegasys®).[139]

Apart from those compounds many other studies relying on NHS conjugation can be given as examples, such as PEGylation methods of different proteins and hormones[37,140–142] (even providing an orally bioavailable form of insulin);[143] some antibody modification strategies, either for surface immobilization,[144,145] or for ADC's development (Genentech's TDM1 and Immunogen's Mylotarg®);[123,146,147] virus's capsids modification[148,149] and, finally, incorporation of different chelating agents for bioimaging, either for nuclear imaging with a sarcophagine ligand or polyaminopolycarboxylic ligands,[150,151] as for optical imaging, with quantum dots or rhodamine derivatives.[152,153]

Reductive amination is one of the earliest alkylations procedures of lysines described. Aldehydes and ketones are known to react selectively with primary and secondary amines to form Schiff bases. However these imines are considered to be unstable in aqueous media, suffering hydrolysis and thus, regenerating the starting materials. Usually the imine formation is enhanced by alkaline pH values, but still a reducing agent is required to stabilize this alkylation (Scheme **21**). Reducing reagents known to perform this transformation are: sodium boro hydride ($NaBH_4$) or sodium cyanoboro hydride ($NaBH_3CN$), which react preferentially at pH ~9 and ~7, respectively.[30]



Scheme **21** – Lysine modification – Aldehydes and subsequent reduction

In the last century, several improvements of this reaction have been made, leading to mild reducing conditions and the main advantage of this reductive alkylation, which is the lack of cross-reactivity. This modification will be discussed in further detail in **Chapter II**. where the selectivity issue will be thoroughly discussed. Similarly to the other amine modifying reagents, this strategy was also used in important applications that induced surface immobilization,[154,155] artificial glycosylation[42,156] and PEGylation of

several biomolecules,[157,158] the synthesis of a FDA/EMA approved drug (Neulasta®), which is a PEGylated pro-drug indicated to decrease the risk of infection for patients under chemotherapy, stimulating neutrophile generation.[159]

Two very interesting studies based on Schiff base formation and subsequent stabilization through catalyzed reduction or 6π-azaelectrocyclization show an important evolution for the reductive alkylation strategy. These reports showcase the potentialities of this selective reaction with lysines or *N*-terminal function, where the overall charge state of the protein is maintained (minimizing effects in protein solubility) and competitive hydrolysis pathways that occur with some activated esters are avoided.

First, Francis and McFarland present an evolution of the wide-known reductive amination by replacing the harsh reducing reagents with an efficient water-stable iridium complex (Figure **1**), that can achieve the same results as hydrides at room temperature and at a wider pH window (from 5 to 7.4). According to the authors, this reaction has shown a wide tolerance in terms of protein and aldehyde component, however this complex performs poorly when ketones are used.[95] This study will be discussed in further detail in **Chapter II.** since it was one of the core studies that support the work described in this thesis.

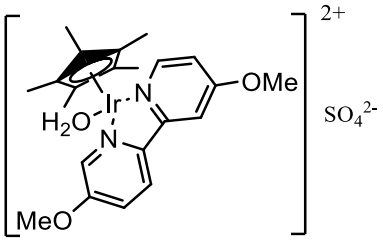
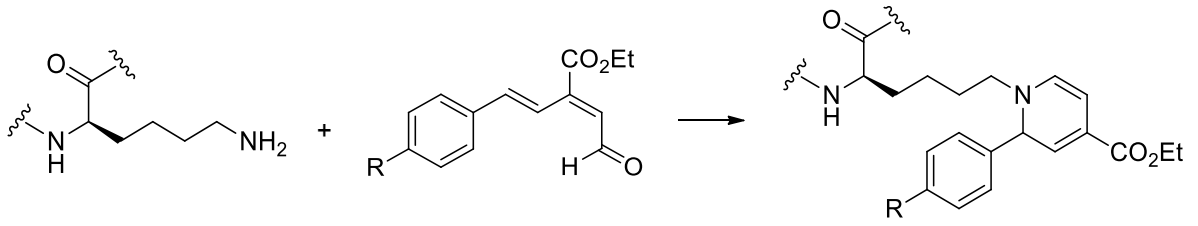


Figure **1** – Iridium Catalyst for Reductive Amination

Another study that presents a major advantage for reductive amination is based in Katsumara's findings that described aldehyde terpenoids as selective reagents to modify protein's amine groups.[160] In 2008, Fukase *et al.* presented a rapid 6π-azaelectrocyclization, as a way of stabilizing a Schiff base formed between lysine and unsaturated (*E*)-ester aldehydes (Scheme **22**). This new lysine-based labeling method allowed the modification of several biomolecules and the incorporation of several bioimaging agents, within 30 minutes, at room temperature.[161] Later that year, the same group presented an evolution of their work by reporting a site-selective modification of Lys137 attained by directing reactive groups to a specific site using a small-molecule ligand of human serum albumin (HSA). In the end, the auto-oxidation of the 1,2-dihydropyridine led to the hydrolysis of the ester linkage connecting this ligand.[162]



41 **51** **52**

Scheme **22** – Lysine modification – Unsaturated (*E*)-ester aldehydes

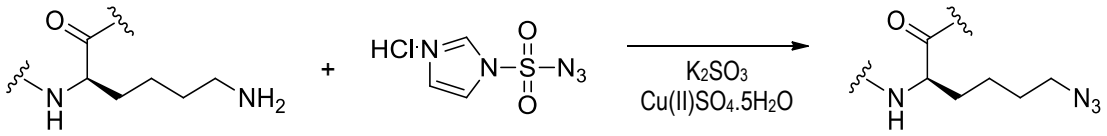
Site-selective strategies to modify lysine residues are of paramount importance, though only a very few number of studies to achieve this have been reported. This is justified due to the availability of multiple lysine residues on a protein, which upon modification often leads to heterogeneous mixtures of conjugates.[36] Apart from the study just described, a study from Weil *et al.* is worth highlighting since it reports a site-selective lysine modification *via* kinetically controlled labeling. According to the authors, the NHS methodology can be developed towards synthesizing monofunctionalized conjugates.[163]

In this revolutionizing study, Weil *et al.* describe a "kinetically-controlled protein labeling" (KPL) approach, where common commercially available biomolecules (ribonuclease A, lysozyme C and somatostatin) are monoconjugated through an established methodology of 6 steps, namely:

1- Bioconjugation step (conjugation of biomolecule with the NHS ester)
2- Quenching step (addition of ethanolamine to quench unreacted NHS ester)
3- First biotin-removal step (filtration with a 1.5kD cut off to remove biotin-small molecules)
4- Protein recovery step (loading mixture to a monomeric avidin resin column to recover excess of native protein)
5- Affinity elution step (eluting this column with biotin to recover the monobiotinylated protein)
6- Second biotin-removal step (another filtration to purify the final product)

According to the authors this KPL approach leads to a more selective labeling since more reactive and accessible residues should react faster. Thus, the authors provide a preliminary computational study in which it was highlighted the importance of solvent accessibility, as well as interaction with adjacent amino acids to justify the differences in reactivities from K1 of ribonuclease A and lysozyme C. Furthermore, the synthesis of bioorthogonal linkers bearing a terminal alkyne and an azide were incorporated in ribonuclease A *via* this "chemical" approach, and then subsequently reacted with an azidocoumarin (which is a very useful fluorophore for click labeling since it only emits fluorescence after the triazol ring formation) and a rhodaminephosphine, respectively.[163,164]

Although the latest study to be mentioned did not start as a site-selective modification of lysine, like the reductive amination from Francis and McFarland, it introduces a novel metal-mediated modification of lysine that can be very useful in a near future. In this case, an azide is introduced through direct diazo transfer to lysine, showing an alternative for Dha generation, since it introduces a bioorthogonal agent that can be used for extra modification, without altering the native protein's surface with long linkers. Thus, van Hest *et al.* reported an insertion of azide groups in proteins through diazo's transfer with copper (II), in aqueous medium. The introduction of an azide function is a very interesting protocol, since the azide is the partner for invaluable Huisgen [3+2] cycloadditions (click reaction). Therefore, an alternative to avoid genetic incorporation of non-natural amino acids is presented through this direct diazo transfer to lysine residues, using imidazole-1-sulfonyl azide hydrochloride (**53**) and copper (II), which was successfully used to modify the enzyme horseradish peroxidase and an engineered red fluorescent protein from *Discosoma striata*.[165] Later, the authors acknowledge the concern of modifying all amino functions of a proteins and, thus, reported a protocol without copper at lower pHs that improved the method to a site-selective procedure where only a single azide function was introduced at the *α*-amine of *N*-terminal function.[166]



41 **53** **54**

Scheme **23** – Lysine modification – Imidazole-1-sulfonyl azide

In conclusion, numerous reagents have been reported to selectively modify lysine residues, since, like cysteine, lysine possesses chemical advantages that grant some level of selectivity in the presence of other chemical functionalities. Although the modification of this amino acid has been slightly neglected due to the high heterogeneity that the most common methods (acylations and alkylations) often lead to, recent reports that described site-selective modifications of lysine residues, either by kinetic labelling as much as for ligand-affinity guidance show great promise in a near future.

I.2.1.3. Other Natural Amino Acids

By contrast to cysteine and lysine, the other canonical amino acids have been scarcely modified, although some present promising advantageous in terms of site-selective modifications.

The acidic residues have been mostly targeted *via* coupling with amines through carbodiimides (though cross-linking is a common limitation of this strategy).[91] Some strategies have been pursued in order to circumvent this limitation of modifying acidic residues with carbodiimides and amines, such as the use of hydrazides, hydrazines or weakly basic amines (that react preferentially with activated carboxylic acids), as well as controlled pH.[167–169]

Tyrosine has a phenolic side chain, which gives an amphiphilic nature to this residue , determining its common localization in a protein (partially buried in the surface of proteins).[170] This residue has been modified based on two different strategies involving aromatic electrophilic substitution and metal-mediated modifications.[171,172] Due to the nature of the side chain (activated aryl group) the phenol *ortho* position can be modified using electron-rich anilines and aldehydes in a selective three-multicomponent Mannich reaction,[170,173,174] or using iodination or diazonium-coupling reagents (diazonium salts or diazodicarboxamides) to perform hetero-Diels-Alder reactions.[167,171,175] Barbas III *et al.*, using diazodicarboxamides, was able to modify chymotrypsinogen A and a therapeutic antibody herceptin without changing its affinity to Erb B-2.[171]

Tryptophan is the rarest amino acid among all proteinogenic amino acids. Still, approximately 90% of proteins (depending on the species) possess, at least, one Trp in their constitution.[176] Metabolic reactions or disorders of tryptophan may lead to pathologies, such as cataract, emphysema, rheumatoid arthritis, blue diaper syndrome and purple urine bag syndrome.[177–179] There are two main approaches concerning the selective modification of Trp residues. One is based on the formation of metallocarbenoids from diazo species catalyzed by dirhodium (II) complex[177,180,181] and the other reaction takes advantage of the high reactivity between secondary amines and dialdehydes.[176,182]

As far as site-selective modifications of the other 18 proteogenic amino acids goes, few have been targets of modification. Nevertheless, tyrosine seems to be the most promising residue for this purpose. The reported aromatic electrophilic substitutions and metal-mediated modifications of tyrosine show a high selectivity regarding other amino acids, though some procedures can be somewhat toxic to some biological systems as well as having a limited applicability.

I.2.2. Modification of Terminal Amino Acids

In terms of general site-selective modification of native proteins, apart from targeting accessible disulfide bonds, specific modification of terminal functions of a protein can be seen as a promising approach that avoid resorting to genetic engineering.

Proteins' terminal chains have some specific reactions to modify them, either by exploring the reactivity of specific terminal amino acids, as by modifying them based on their singularity. Targeting the terminal amino acid offer several advantages when comparing with other exposed residues. This includes a high flexibility and solvent accessibility, rare or none implication in biological activity and single function (of each) per protein.[183] Their resemblance with side chains of some amino acids justifies their limited application, though a restriction of modifications towards specific terminal amino acids can increase selectivity. The reactions that are performed in these terminal functions can be divided in: modifications that do not require a specific amino acid to occur, in opposition to the ones that do require a specific amino acid. It is also important to mention that these modifications play a huge role in peptide coupling strategies, since modifications that lead to general amidations are of crucial importance to chemically combine peptides.

Regarding modifications of general terminal endings, two scenarios can be considered. In the case of *C*-terminal functions, they are hard to selectively modify without resorting to recombinant technology.[183] The *N*-terminal function, on the other hand, is easier to modify because it is distinguishable from other amines based on its different pKa.[184–187]

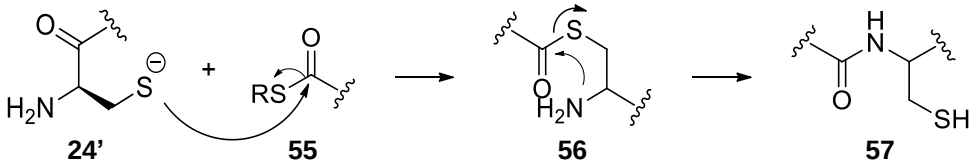
Different amino acids (or sequences) can be mentioned as being required on a protein *N*-terminal ending for a modification to proceed. As examples aspartic acid,[184] serine,[188] proline,[189] cysteine,[77] alanine, glycine/serine and proline sequence,[190] among others (that can be found in an excellent review from Bertozzi and Sletten)[91] were used to perform site-selective modifications.

Nevertheless, the most crucial work performed in terminal modifications of specific *N(C)*-terminal peptides and proteins is the one that leads to native bonds between two amino acid chains. This work has been decisive for the recent development of synthetic biology, since it is a vital part for chemical protein synthesis.

Although it is not the central topic of this thesis, due to its relevance in terms of applications, a brief discussion of chemical ligation of peptides and proteins will be presented. Since this methodology usually requires specific *N*-terminal and *C*-terminal functions to be performed, it can be included in this topic, though it can not hardly be considered as a modification of natural proteins in their native state.

Nevertheless, it finds in peptide and protein synthesis a huge field of applications that shouldn't be disregarded.

Chemical ligation is a field of research that has its foundations on a chemical reaction between a *N*-terminal cysteine and a *C*-terminal thioester, which was first described in 1953, by Wieland *et al.*, where the *S,N*-acyl shift is highlighted for this transthioesterification.[191] From this study, in 1994, Kent *et al.* developed a methodology to combine different unprotected peptides that fulfilled the aforementioned requirements for their terminal functions. Stephen B.H. Kent named this process native chemical ligation (NCL, Scheme **24**) and since then it has been an extensively used strategy for the total chemical synthesis of peptides and proteins.[192]

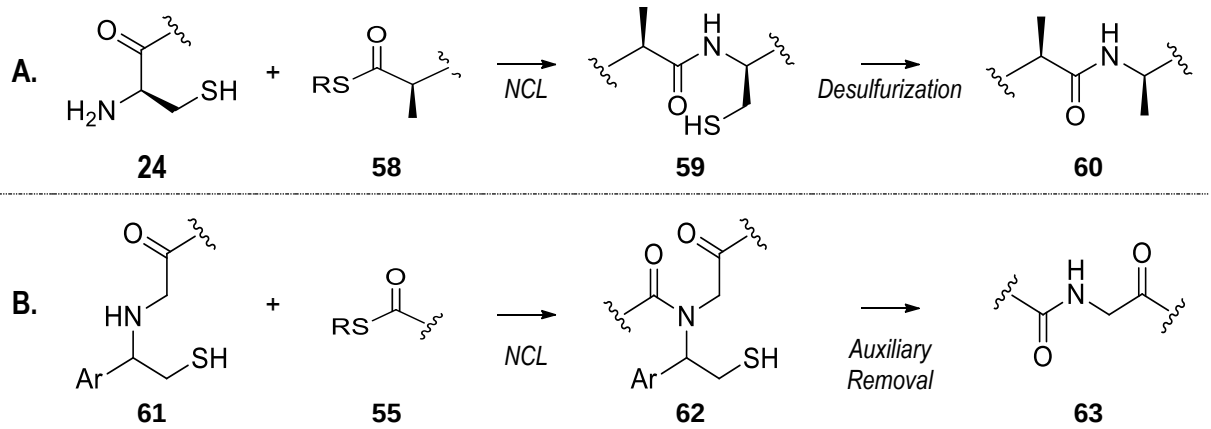


Scheme **24** – *N*-terminal cysteine modification – NCL mechanism

In the following years, much research has been done in NCL methodology using synthetic chemistry to elucidate the molecular basis of protein function, and particularly enzyme catalysis. For instance, *in vivo* compatibility was reported, allowing to promote protein's biotinylation, solid support immobilization (maintaining protein's biological activity) and biosignaling.[193]

Stephen Kent disclosed several studies that improved this technology and demonstrated its vast applicability. In 2004, Kent *et al.* showed, for the first time, the total synthesis of a folded protein (crambin) by combining three unprotected peptides through NCL. In this study, 1,3-thiazolidine-4-carboxo moiety was introduced as a protecting group for *N*-terminal Cys, in order to guide the right sequence of a protein through a protection/deprotection system. This deprotection was achieved by using methoxyamine.[194] In 2005, Kent and co-workers reported the total synthesis of ubiquitin.[195] A year later, after an extensive study of the reaction's mechanism, a relationship between the thiol's pKa and its reactivity in NCL was established,[196] Kent *et al.* reported a fully convergent protein synthesis based on kinetically controlled ligation based on the use of different thioesters.[197] In the same year, a method of direct on-resin synthesis of protein through NCL was described, highlighting a fast production of *α*–thiophenylesters peptides.[198] Among other reports of total proteins synthesis, in 2008, the same group, reported the total synthesis of the enzyme ribonuclease A and its biochemical characterization.[199]

In 2009, Stephen Kent wrote an excellent review on this topic, in which is discussed the most important limitation of this methodology which is to proceed only with a *N*-terminal cysteine residue. In this review, the author also emphasized the attempts on chemically expanding NCL to *N*-terminal non-Cys unprotected peptides, which were based on two different methodologies (Scheme **25**).[200]



Scheme **25** – Methodologies to expand NCL to *N*-terminal non-Cys unprotected peptides.
A.- desulfurization of cysteine; B.- Mimetics of cysteine (*N*(α)-auxiliaries)

One being the desulfurization of cysteine in the NCL product, enabling the production of Ala peptides where a cysteine would originally be (Scheme **25A**). This reduction procedure with palladium or nickel was first described by Dawson and Yan, but it has been extended to other β-mercapto amino acids, like phenylalanine.[200] Later, Danishefsky and Wan, concerned with the lack of chemoselectivity of the metal-mediated reduction, developed a procedure using TCEP based reduction, applying it to generate peptides with alanine or valine in the final products.[11] Later on, trifluoroethanethiol was also described as an additive for the same purpose leading to an aspartic acid and an alanine instead of their β-mercapto amino acids.[201] Recently, other amino acids (like tryptophan, arginine or proline) were used as a mercapto amino acids to perform the NCL and then reduced.[202–204]

The second attempt to expand NCL to *N*-terminal non-Cys peptides is based on using thiol containing *N*(α)-auxiliaries that mimic the presence of cysteine and that can be selectively removed once the ligation has occurred (Scheme **25B**). This strategy was first reported by Macmillan *et al.* and ideally it could be applied to any terminal amino acid, however, these molecules tend to be highly sensitive to the nature of the amino acid and to substitutions on the *α*-carbon atom of the thioester, and for those reasons proved to be less efficient than regular NCL.[205]

On the other hand, in terms of the *C*-terminal thioester for NCL, its requirement has also been discussed and, among other developments, in 2007, Danishefsky *et al.* replaced this function for a phenolic ester with an *ortho* protected thiol group with a simpler synthetic pathway.[206]

Early applications of the NCL were based on the formation of microproteins, cyclic proteins, neoglycoproteins and artificial proteins with non-natural amino acids (NNAA) or with artificial post-translational modifications. Furthermore it was one of the most used tools in order to combine different peptides through solid-phased peptide synthesis (SPPS).[207]

From this methodology, others have emerged to enhance NCL's applications, combining chemical and biochemical tools to achieve better results.

Based on the resemblance of NCL's mechanism and the natural mechanism of protein self-splicing (where a domain of a protein, referred as intein, is extruded in a post-translational process), two other techniques were developed to make use of recombinant proteins. First, expressed protein ligation (EPL) was described, which is based in a chemical process between a recombinant protein (intein) with a thioester and a chemically synthesized *N*-terminal cysteine peptide, that can occur under a wide range of reaction conditions. And then, the protein-*trans* splicing (PTS) technique was reported, where, in contrast, has to be performed under naturing conditions since the process involves the functional reconstitution of a split intein. This splicing occurs when the two complements of inteins meet each other, since a part of the split intein is fused to the target recombinant protein and its complementary portion is connected to another synthesized peptide (Figure **2**).[91,191,208]

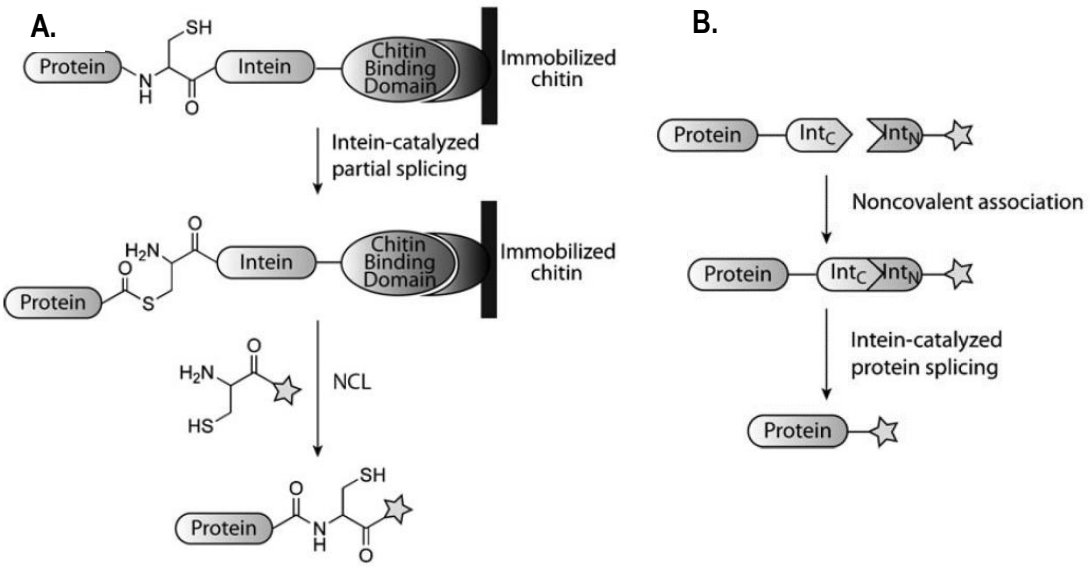


Figure **2** – Expressed protein ligation (EPL, A) and protein trans-splicting (PTS, B) mechanism. Image adapted from a review from Sletten and Bertozzi[91]

Apart from these ingenious strategies directly descendants of NCL, other ligations have been purposed to combine peptides under naturing conditions. Despite the fact that some ligations are more general than others five examples have been successfully implemented (Table **2**):

1 – Traceless Azide-Staudinger Ligation – reaction between an azide and a thioester that uses a thiophosphino auxiliary that yields an amide product without any residual atoms;[209]

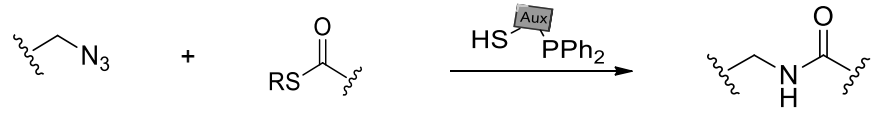
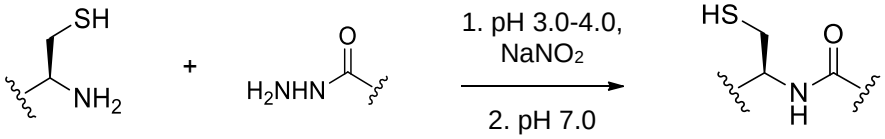
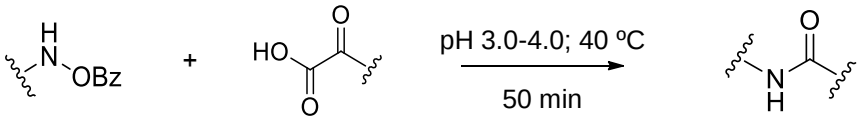
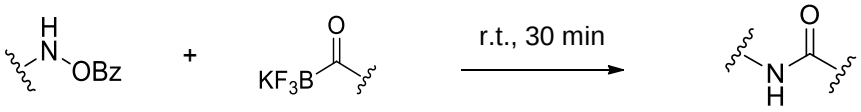
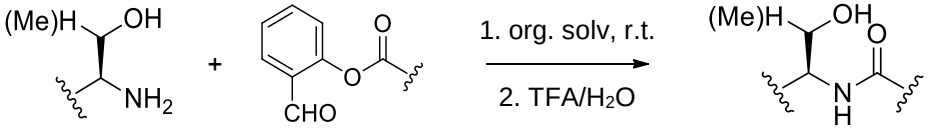
2 – Peptide Hydrazides Ligation – reaction between a *C*-terminal peptide hydrazide and a *N*-terminal cysteine to yield a native peptide bond, by using an oxidant in a first step and a thiol in the final step;[210]

3 – *α*-Ketoacid-hydroxylamine (KAHA) Ligation – decarboxylative amidation of a hemiaminal intermediate produced from an *α*-keto acid and a *N*-alkylhydroxylamine;[211,212]

4 – Acyltrifluoroborate-hydroxylamine (ATHA) Ligation – improved KAHA ligation with increased reaction speed and nonacidic conditions between *O*-benzoyl hydroxylamines and acyltrifluoroborates under aqueous solvents;[213]

5 – Serine and Threonine Ligation (STL) – reaction between a *C*-terminal salicylaldehyde ester and a *N*-terminal serine or threonine that forms an imine with its *α*-amine group and leads to an irreversible amide bond formation (through a 1,5 *O*→*N* acyl shift).[214,215]

Table **2** – Alternatives for Native Chemical Ligation

Ligation	Reaction
Traceless Azide Staudinger	
Peptide Hydrazydes	
α-Ketoacid-Hydroxylamine (KAHA)	
Acyltrifluoroborate-hydroxylamine (ATHA)	
Serine and threonine (STL)	

Apart from individual examples of modification of general terminal endings, functionalization of specific terminal endings of peptides and proteins tend to be the most followed strategy within these parameters. Although this methodology is highly selective its applicability is quite scarce without resorting to genetic engineering. In that sense, introduction of non-natural amino acids is the competitive methodology in terms of selectivity.

I.2.3. Modification of Non-natural amino acids

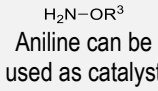
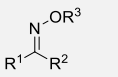
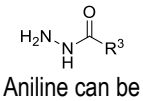
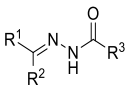
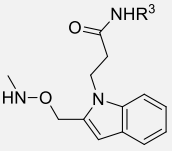
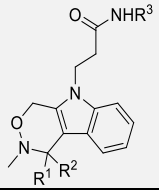
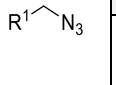
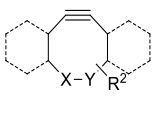
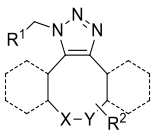
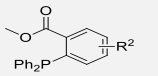
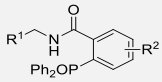
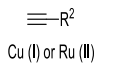
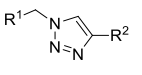
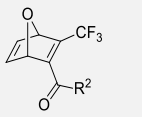
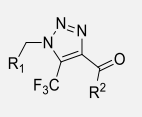
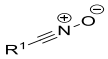
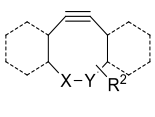
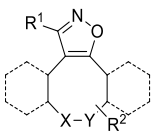
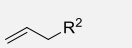
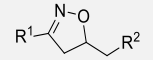
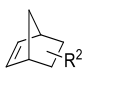
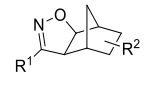
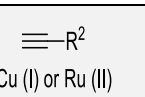
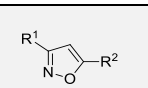
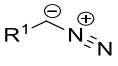
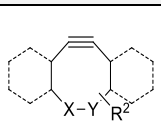
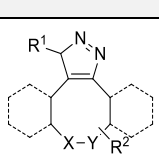
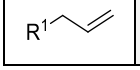
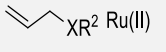
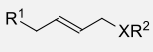
In terms of site-selective modification of peptides and proteins, it is practically impossible to compete with the selectivity that the introduction of non-natural amino acids (NNAA) permits. The incorporation of a chemical functionality that has no match in biology and exhibits a specific chemical reactivity, allows unprecedented levels of selectivity and specificity. This methodology faces a major limitation related with the incorporation of the NNAA. This has been achieved *via* translational or semi-synthetic incorporation.[1]

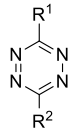
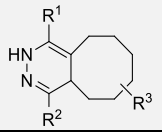
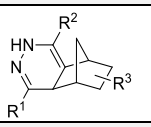
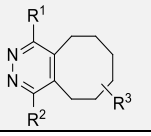
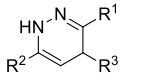
Translational incorporation is based in different methods, such as: use of auxotrophic strains, stop codon suppressor tRNA, orthogonal aminoacyl-tRNA synthetase, non-natural base pairs or by expanding the size of codons. In contrast, semi-synthetic incorporation is mainly based in a single methodology named solid-phase peptide synthesis (SPPS).[1,27] Excellent reviews, as well as two recent letters from Schultz *et al.* and Liu *et al.*, can be consulted to show the diversity of NNAA that have been incorporated throughout the years.[1,216–222]

Incorporation of NNAA led to the possibility of using chemical species that allow reactions that neither interact with nor interfere with biological systems. In 2003, Bertozzi *et al.* described these reactions as bioorthogonal reactions, though some reactions with those properties have been described prior to that term. According to these authors, the functional groups for this type of reaction have to respect two major requirements, which are the inertness towards biological moieties and exclusive reactivity against its counterpart under biocompatible conditions. Moreover, for *in vivo* applications, they also shall be nontoxic to cells and organisms.[223]

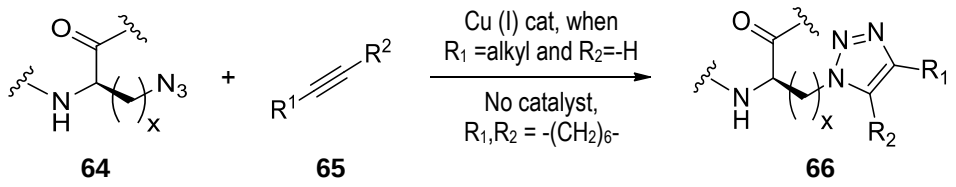
Despite the difficulties inherent with the development of such biocompatible reactions, several bioorthogonal reactions have been described, as it is shown in Table **3** (adapted from two recent reviews).[135,224] One or both of the reaction's counterparts have already been directly incorporated into proteins or, in alternative, indirectly attached by means of chemical modification of natural amino acids, as it has been described in **I.2.1. Modification of Natural Amino Acids**. From these reactions, the most recurring modifications rely on cycloaddtions between azides and alkynes (whether terminal or cyclic), condensations of aldehydes/ketones and some metal-mediated reactions with halogenated amino acids.

Table **3** – Bioorthogonal Reactions

Chemical Reporter	Reaction counterpart and catalyst	Product	Reaction Type
	 Aniline can be used as catalyst		Aldehyde/ ketone aminooxy condensation
	 Aniline can be used as catalyst		Aldehyde/ ketone hydrazide condensation
			Aldehyde/ ketone condensation Pictet-Spengler
			Strain-promoted Azide-Alkyne Cycloaddition (SPAAC)
			Staudinger Ligation
	 Cu (I) or Ru (II)		Copper Azide-Alkyne Cycloaddition (CuAAC)
			Tandem [3+2] Cycloaddition retro-Diels-Alder
			Strain-promoted Azide-Nitrile Oxide Cycloaddition (SPANOC)
			SPANOC
			SPANOC
	 Cu (I) or Ru (II)		SPANOC
			Strain-promoted 1,3-dipolar Cycloaddition
	 Ru(II)		Cross-Metathesis

Chemical Reporter	Reaction counterpart and catalyst	Product	Reaction Type
			Inverse Electron-Demand Diels-Alder (iEDDA)
			iEDDA
			Strain-promoted [4+2] Cycloaddition
			iEDDA
			[4 + 1] Cycloaddition reaction
			iEDDA
			Strain-Promoted Alkyne–Nitrone Cycloaddition
	UV light		Photoinduced Cycloaddition reaction
	UV light		Photoinduced Cycloaddition reaction
	UV light		Photoinduced Cycloaddition reaction
			[2+2+2] Cycloaddition reaction
			Click hetero-Diels–Alder Cycloaddition
	Pd(II)		Sonogashira Coupling
	Pd(II)		Suzuki Coupling

Of these cycloadditions between azides and alkynes, 1,3-dipolar Huisgen cycloaddition is one of the oldest reactions described and meets the majority of criteria to selectively modify a protein. Both counterparts have been incorporated into proteins as analogues of different amino acids, such as Phe, Tyr, Ala and Met.[10,15,225–227] As it is easy to comprehend, the labeling agent has the complementary termination for such reaction to occur, whether is mediated by a metal (copper (I)), when a terminal alkyne is one of the counterparts, or without metals, when cyclooctyne is the reaction's counterpart (where ring strain release acts as the driving force of this reaction). [10,27,225–227] Although the final product is always a 1,4-disubstituted triazole (**66**), the name of the reaction depends upon the driving force of the reaction, which can be copper – copper azide-alkyne cycloaddition (CuAAC) – or, ring strain release – strain-promoted azide-alkyne cycloaddition (SPAAC). In Scheme **26**, an example of such reaction is presented, where the azide was introduced to a protein and the labeling agent has an alkyne in its constitution.[135,224]



64 **65** **66**

Scheme **26** – NNAA Modification – Azide-alkyne cycloaddition

When incorporating aldehydes or ketones to a peptidic based structures, a series of selective condensations can be made with these functional groups. Of those, the most broadly used is the reaction with hydrazides, which are chemical species that can be added to fluorescent probes or biomolecules. For instance, Schultz *et al.* selected a translational incorporation of NNAAs and reported a successful selective labeling, *in vivo*, of *LamB* protein with hydrazide-fluorescein.[228]

For reactions with incorporated halo-amino acids, there are two different approaches worth mentioning. One, which is not bioorthogonal since the reaction's counterpart is a nucleophile that can react with other electrophiles using a simple bimolecular nucleophilic substitution (S_N2) as its foundation,[229] and another that takes advantage of palladium (II) to perform a Suzuki coupling between a boronic acid and a halogenated amino acid which allows the conjugation with several arenes.[230]

In recent years, with the significant development of bioorthogonal reactions, an increased concern has been directed to the kinetics of these reactions that can limit their effectiveness under highly dilute reaction conditions, such as the ones required for *in vivo* applications.[231] This is the guideline that is promoting the development of new bioorthogonal reactions, where iEDDA and SPAAC reactions present the fastest reactions rates (Figure **3**).

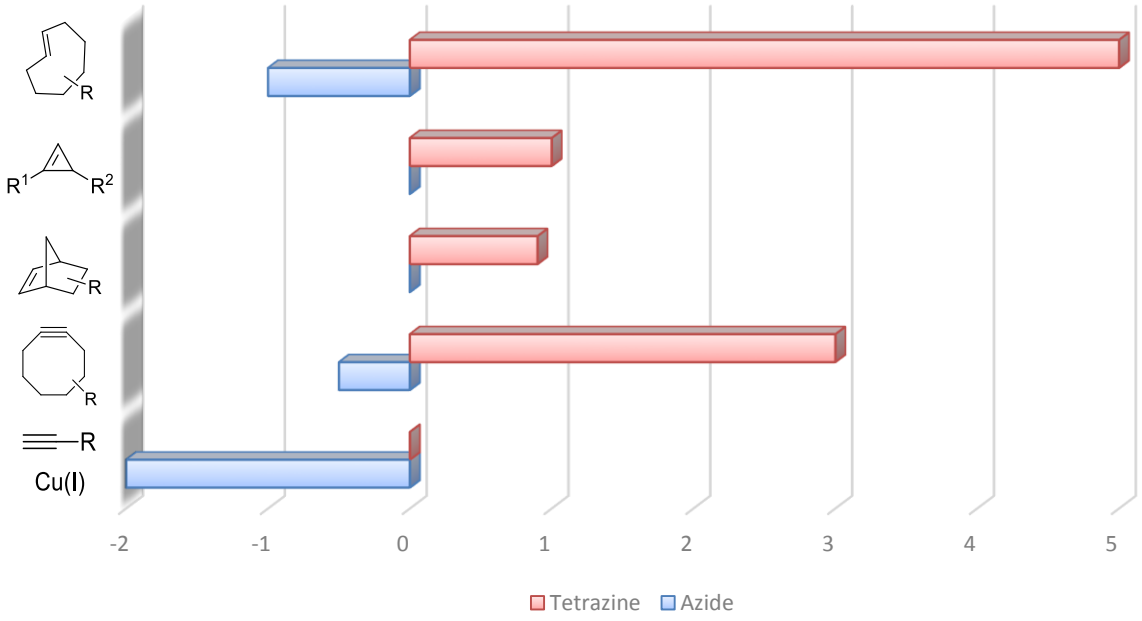


Figure **3** – Reported reaction rates of tetrazines and azide with different counterparts. XX values are logaritmic values of second order rate constant in $M^{-1}s^{-1}$. Figure adapted from two reviews.[224,231]

Apart from the aforementioned functionalities that enable bioorthogonal reactions, there are also other functional groups that have been incorporated into proteins that promote selective reactions with certain counterparts. The incorporation of selenium amino acids that lead to Dha through oxidative elimination is the best example of such methodology, since it introduces an electrophilic species otherwise absent from a peptidic structure. *Seebeck and Szostak* successfully incorporated selenium amino acids that were genetically encoded.[232]

In terms of site-selective modification of proteins, the simplification of non-natural amino acids incorporation is promoting this field of research into one of the most promising areas of bioconjugation. Although there are still some conservative doubts regarding the use of these abiotic species mainly due to unexpected changes that these functionalities can acquire in a recombinant protein or in a living organism, a major growth of this area is to be expected in a near future as an alternative of the well-established modification of natural amino acids (mainly, cysteine and lysine).

General Objectives of the Thesis

In recent years, the site selective functionalization of proteins entered into the limelight of synthetic organic chemistry and many research groups are now actively looking for new ways to create bonds with proteins. In **Chapter I.** an overview of the state of the art for this area of research was given and based on such information, the objectives established for this project will now be presented.

The main goal of this project is the development of several new reagents for manipulation of non-recombinant proteins. To achieve this objective, a selective and reversible modification of primary amines was studied, looking to overcome some of the gaps in the present state of the art.

Based on the fact that aldehyde/ketone condensation has a great advantage among other lysine modifying agents, since it presents lack of cross-reactivity in a wide pH range, this reaction was taken as a starting point. However, the reported studies of this methodology indicated the instability of the final product as a drawback that required a subsequent reduction to form a stable protein modification.

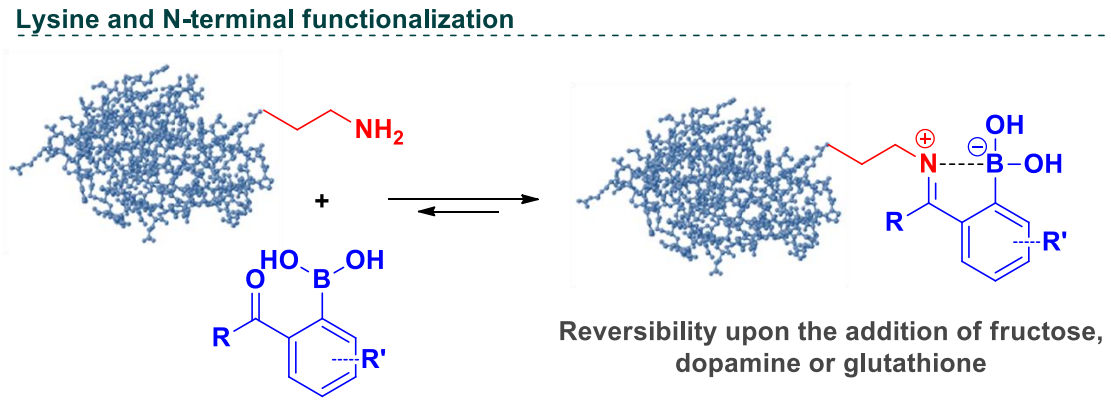
To overcome this limitation we envisioned to stabilize the imine linkage towards hydrolysis and, at the same time, have a tunable reversibility. With this purpose in mind, a search of different boronic acids' based reagents was developed to have a selective (with a controlled reversibility) primary amine modification of biomolecules, which will be discussed in **Chapter II**.

Once the conjugation was fully described, the synthesis of derivatives with specific chemical properties was crucial. Therefore, in **Chapter III**. the search to develop an adjustable core structure of the novel modifying agent will be presented. Followed by the description of the installation and biological applications of fluorescent labels, "clickable", PEGylating and cytotoxic agents in **Chapter IV**.

Finally, in **Chapter V.** an ongoing project will be presented, in which, chemical improvements to the core structure are being developed to synthesize a molecule that can have a more controlled reversibility upon specific pH conditions.

Chapter II

Searching Conjugation with Controlled Reversibility



ABSTRACT

Protein modification has entered the limelight of chemical and biological sciences, since, by appending small molecules into proteins surfaces, fundamental biological and biophysical processes may be studied and even modulated in a physiological context.

In this chapter, a new strategy to modify the lysine's ε-amino group and the protein's *N*-terminal will be presented, based on the formation of stable iminoboronates in aqueous media. This functionality enables the stable and complete modification of these amine groups, which can be reversible upon the addition of fructose, dopamine, or glutathione. A detailed DFT study is also presented to rationalize the observed stability of the iminoboronate constructs toward hydrolysis.

Chapter II. Searching Conjugation with Controlled Reversibility

II.1. Introduction

II.1.1. Reductive Amination

As mentioned in **Chapter I.** reductive amination is one of the available tools to modify lysine residues of natural proteins. This procedure is based on a selective reaction between a carbonyl group and a primary or secondary amine to form Schiff bases that are hydrolytically unstable and, thus, need to be reduced. The lack of cross-reactivity is one of the most appealing aspects of this methodology, although the highlighted instability and subsequent requirement for reduction constitutes important limitations that needed to be overcome.

Two different strategies were developed in order to overcome this limitation: one being the development of water-soluble iridium catalysts for transfer hydrogenation (to avoid harder reducing agents) and the other being based on the development of reagents that stabilized this unstable linkage, once it is formed, mainly through intramolecular reduction.

The study of Francis and McFarland that developed water-soluble iridium catalysts has been briefly described in **I.2.1.2 Lysine**,[95] however some features of this work remained unstated, namely the activation of the aforementioned catalyst to reduce the imine, as it is illustrated in Figure **4**, which explains the required stoichiometric amount of sodium formate. The authors classified their strategy as being able to functionalize proteins with similar efficiencies to NHS-ester and sodium cyanoborohydride-based techniques, without using water-sensitive or harsh reagents. This study served as basis for the methodology described in this chapter, by delineating reaction conditions and outlining model proteins that can be used to evaluate a lysine modifying agent.

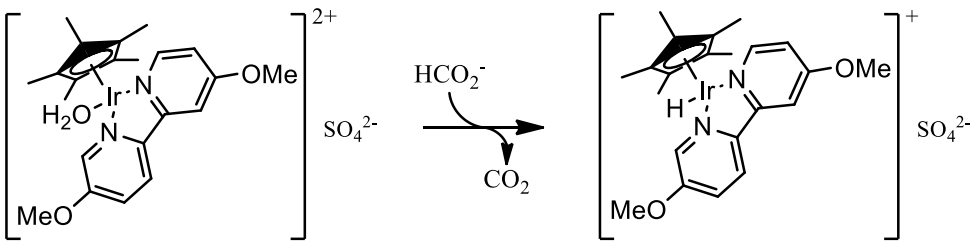
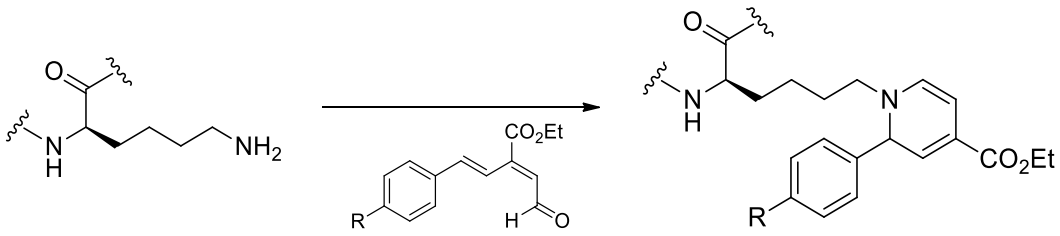


Figure **4** – Iridium's catalyst activation

Apart from this study of transfer hydrogenation, a more common strategy to take advantage of this selective reaction is to use specific aldehydes or ketones that lead to a stable and irreversible final product through intramolecular rearrangements, even in aqueous media. An example of such approach is the use of unsaturated *(E)*-ester aldehydes (**51,** Scheme **27**).



41 **51** **52**

Scheme **27** – Lysine modification – Unsaturated (*E*)-ester aldehydes

In 1999, aldehyde terpenoids were described as agents to modify lysine residues that inhibited bovine pancreatic phospholipase A_2, by irreversible formation of dihydropyridine derivatives.[160] Using the same core structures, Fukase *et al.*, in 2008 reported unsaturated *(E)*-ester aldehyde derivatives, which led to the development of an efficient PET protocol.

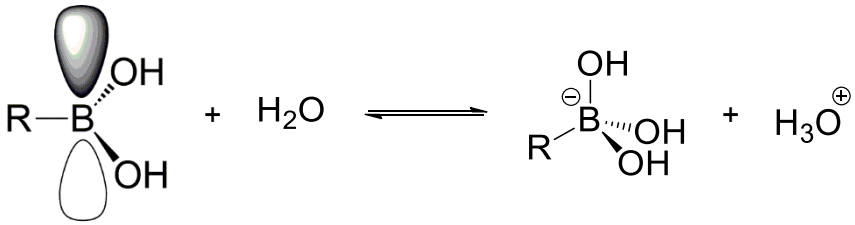
Once the mechanism of the above-mentioned procedure was explained, the authors synthesized different derivatives that could be used for bioimaging applications. On one hand, having the same core structure, the authors synthesized rhodamine and coumarin derivatives available for optical fluorescence imaging, on the other hand, the same authors, developed a derivative of a chelating agent (dota-O-succinimidyl ester), which was used to chelate gadolium (^{68}Ga). This compound was then used for nuclear imaging by conjugation with somatostatin, showing images that grounded the accumulation of this conjugate in the liver, kidney and pancreas, as it was expected.[161] Later that year, the same authors also reported a site-selective modification of Lys 137 of HAS by ligand directing strategy.[162]

Inspired by these results, which reported a new lysine-based labeling method with Schiff bases as intermediaries, we have developed a method in which the Schiff base would be the final product, once it was stabilized by another functional group (a boronic acid) present in the same molecule. This strategy would have an advantage in that we would be able to have a controlled reversibility mechanism induced by certain molecules. If successful this strategy would have promising applications in terms of drug delivery. This hypothesis is majorly based on the intrinsic characteristics of boronic acids, which will now be discussed in further detail as a support of what will be described in this chapter.

II.1.2. Boronic Acids

Boronic acids are organic compounds that have a sp^2-hybridized boron atom attached to one alkyl, vinyl or aryl substituent, two hydroxyl groups and a vacant *p* orbital as a consequence of having only 6 valence atoms (**67**, Scheme **28**). These compounds have interesting physicochemical properties that makes them ideal for some biological application, namely, their known low toxicity (with the environmentally friendly boric acid as their ultimate degradation product), their absence in nature and their relative stability to atmospheric oxidation. Boronic acids are also considered to be amphiphilic species since the apolar carbonated chain balances the polarity set through the hydrogen bond donating ability (of their hydroxyl groups) and their Lewis acidity. Due to their unique properties as mild organic Lewis acids and unparalleled reactivity, in the past three decades boronic acids have passed from being rather neglected compounds to valuable synthetic intermediates of modern organic chemistry.[233]

Generally, boronic acids present trigonal planar geometry, where the boron empty orbital is perpendicular to the boron substituents. The exception for this rule is the case of *ortho* susbtittuted aromatic moieties, where the aromatic ring is perpendicular to minimize the steric strain. However, in order to complete boron's octet, boronic acids can also coordinate basic molecules, existing as stable tetracoordinated adducts with tetrahedral geometry. This coordination caused by boron's deficient valence results in a negative charge that is formally drawn on this atom (**69**, Scheme **28**). This complexation explains its unique properties as mild organic Lewis acids in opposition to a usual Brønsted base formed in the equilibrium of hydroxyl groups in water. In other words, the boronic acids ionize water and form hydronium ions by "indirect" proton transfer instead of direct proton removal from their hydroxyl groups.[233]



67 **68** **69** **70**

Scheme **28** – Ionization equilibrium of boronic acid in water

Due to the importance of boronic acids and derivatives, over the past decade, several attempts were made in order to incorporate these molecules in proteins. Even because, this is one of the chemical entities that can act as chemical reporters for bioorthogonal reactions, as it has been mentioned in **I.2.3 Modification of Non-natural amino acids**.

In 2002, through a maleimide reaction it was possible to introduce a fluorescent tag containing BODIPY (boron-dipyrromethene) into a protein, due to the high affinity of this molecule with cysteine.[234] Several years later, different studies were made to fruitfully incorporate similar dyes into a protein by translational modification.[235–237] Nevertheless, these derivatives did not confer many of the advantages that boronic acids could give to biomolecules. So, in 2008, Schultz *et al.* were able to successfully encode a boronate-containing amino acid in a protein of *E.Coli*,[238] as well as, two years later, do the same in *Mycobacterium tuberculosis* organism.[239] Apart from these translational incorporations, another approach uses a semi-synthetic procedure in order to incorporate a boronic acid in the *N*-terminal function of peptides. Like so, Santos *et al.* were able to find selective inhibitors of human ClpXP, which is an ATP-dependent serine protease present in the mitochondrial matrix.[240]

The translational introduction of this molecule into biomolecules allows a bioorthogonal reaction to occur at mild conditions (Suzuki Miyaura cross-coupling) and many experiments were made to perform it either in peptides[241,242] or in proteins.[238,243] However, a chemical incorporation of boronic acids using reagents of chemical modification of biomolecules and subsequent cross-coupling is yet to be proven.

Boronic acids are useful reagents available for synthetic organic chemistry. Their uses expands from traditional organic and organometallic chemistry, in which they are used to establish new bonds, to sugar sensing, material sciences and medicinal chemistry. Notwithstanding, this family of compounds has been almost neglected in what concerns their use as protein modifying reagents.

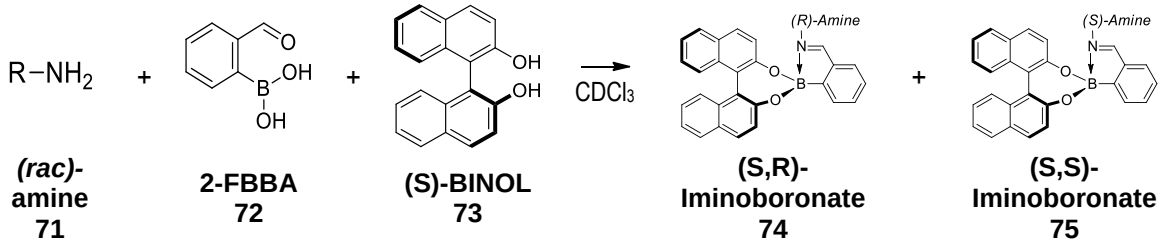
II.1.3. Interaction between Boron and Nitrogen

Aiming at proving our hypothesis that alkylic imine stabilization could occur through interactions with a proximal boronic acid, we initiated this study by evaluating reagents that allow for that N-B interaction to occur. *Ortho*-carbonyl benzene boronic acids have been reported in the literature as synthetic intermediaries to obtain different diazaborines (by reaction with hydrazine),[244,245] macrocycles[246–248] or even as counterparts for Suzuki couplings.[249,250] However, in terms of iminoboronates that highlight the interaction between boron and nitrogen, work from two different authors is worthy of emphasizing. These authors are named Eric V. Anslyn and Tony D. James.

Eric V. Anslyn and co-workers reported a significant number of interesting ^{11}B-NMR, reporting no significant differences between secondary and tertiary amines in terms of complexation geometry and pH dependence.[251] In 2006, important conclusions from these compounds were obtained, namely the

difference between N-B interaction depending on the solvents and on their complexation with diols. If the solvent is aprotic N-B dative bond is present, but on the contrary, if the solvent is protic, the boronate species dominates and a hydrogen-bonded zwitterionic species is the most predominant species. Moreover, if the boronic acid is complexed with a vicinal bifunctionalized, electron-withdrawing substrate to generate a boronate ester, N-B bond formation is promoted, as it is true for solvent insertion due to the increased electrophilicity/Lewis acidity of the boronate.[252] Three years later, the same group performed another study where the N-B bonding was evaluated in opposition to solvent insertion, depending upon the degree of substitution from the amine group. This study proved that there is a slender increase in the N-B dative bonding when using tertiary amines instead of secondary or primary amines, though solvent insertion is the dominant process in all boronate esters formation.[253]

Tony D. James and co-workers developed several applications of 2-formylbenzeneboronic acid derivatives (2-FBBA, **72**) as an element of a three-component chiral derivatizing protocol for enantiopurity NMR determination of different chiral products.[254–258] In these studies, an enantiomeric pure chiral ligand, (S)- or (R)-BINOL (**73**), would be complexed to the boronic acid of 2-FBBA and the amine-based chiral product would form an imine from reaction between the formyl group of FBBA and the racemic amine (Scheme **29**). From these structurally rigid diastereoisomeric iminoboronate esters, a significant difference in the ^{1}H-NMR spectrum of the amines is obtained depending upon the different amine enantiomers.



Scheme **29** – James's protocol for NMR analysis of the enantiomeric purity of different amines

Apart from this very complete study on enantiomeric purity determination of amines, other studies have been reported from this group which were focused on boron and nitrogen interaction. In 2008, James *et al.* reported dynamic covalent self-assembled macrocycles between 2-FBBA and 1,2-amino alcohols, which is a common application for these compounds.[259] A few years later, an interesting review from the same authors mentioned a very attractive dynamic covalent bonding for what we intended from the reversible modification of lysine residues.[260] According to the authors, the Lewis acidity of boronic acids is enhanced whenever boronate esters are formed in the presence of 1,2-, 1,3- or 1,4-diols, in aqueous

media, and, thus the fast and stable bond formation between these chemical species could be the basis of reversible molecular assemblies.

Although these studies of N-B interaction proved to be important milestones for the development of this project, it is crucial to highlight that the majority of the conclusions were taken in aprotic solvent conditions. Moreover, some of these studies gave assumptions based on aminoboronates, which differ from iminoboronates in the C-N bond order. Regardless, all this data concerning N-B interaction gave us momentum to outline our objectives to develop a conjugation system with controlled reversibility.

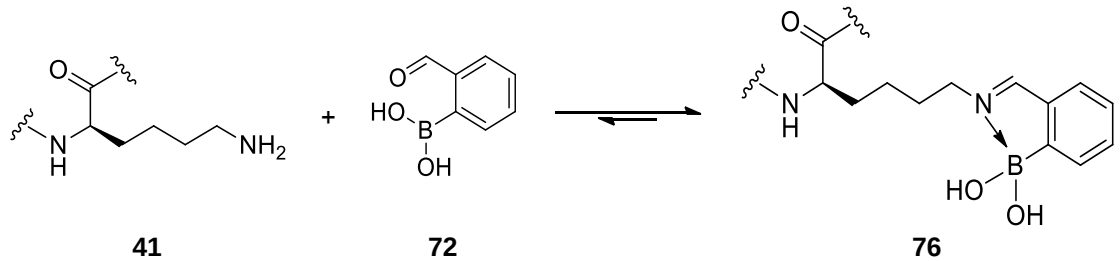
II.1. Goals

This chapter's purpose is to describe all the chemical reactions developed to demonstrate a selective conjugation with alkylic amines in the presence of other functional groups and under naturing conditions. In parallel with the experimental data collected, a theoretical elucidation was developed to highlight the role of the *ortho* boronic acid group in terms of aiding the imine formation and stabilizing the final product.

Moreover, once the conjugation had been well established with several biomolecules, the envisioned reversibility was evaluated using different endogenous molecules that can lead to controlled delivery under certain physiological conditions.

II.2. Exploratory Results and Optimization

We hypothesize in this study that boronic acids, due to a close proximity with the imine would form a potentially stable iminoboronate by forming a N-B dative bond, as depicted in Scheme **30**. In addition, once the conjugation is settled, the disruption of this newly-formed dative bound, would reverse conjugation and regenerate the starting materials. Therefore, the formation of stable imines in aqueous media would allow a direct, selective and potentially reversible strategy to modify lysine residues and *N*-terminus.



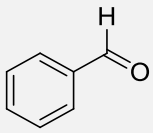
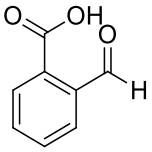
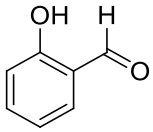
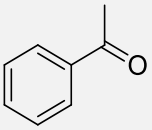
41 **72** **76**

Scheme **30** – Hypothesis - lysine modification with 2-FBBA

To test this hypothesis, we studied the imine formation using 1-butylamine as a model to mimic the ε-amino group of lysine residues. With this amine, we performed several mild reactions with aldehydes and ketones (**77**-**80**) without the boronic acid functionality (under naturing conditions, *i.e.* performed in neat water and mixed for 18 h at room temperature). The results are resumed in Table **4** and will serve as controls for the results obtained with molecules bearing boronic acids.

Considering the dynamic feature of the constructs, we decided not to perform any purification processes that could tamper our conclusions. Therefore, all conversions presented in this chapter are based on the crude ^{1}H-NMR spectra.

Table **4** – Aboronated aldehydes and ketone as control reactions

Aldehyde/Ketone	Conversion of imine formed	Aldehyde/Ketone	Conversion of imine formed
 **77**	20%	 **78**	0%
 **79**	9%	 **80**	0%

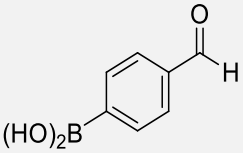
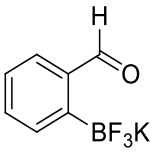
Based on the aforementioned studies, we performed the synthesis of the imine from 1-butylamine and 2-FBBA in organic solvents to have a pure form of the iminoboronate. The imine was obtained quantitatively in dichloromethane. Having the product isolated, finally we could test our hypothesis by adding 2-FBBA to a butylamine aqueous solution. Very pleasingly, the iminoboronate was formed at room temperature in 49% and 52% conversion when the reaction proceeded in neat water or in a phosphate buffer solution (50 mM, pH 8.02), respectively.

Encouraged by these results that dictated an increase of imine conversion to almost the triple of what was obtained with the aboronated species (Table **4**), we carried on our study to comprehend this reaction and improve its efficiency.

First, to highlight the importance of the boronic acid with a vacant *p* orbital in an *ortho*-position relatively to the formyl compound, we test two different boronated compounds that are unable to establish a N-B dative bond. A formylbenzene boronic acid *para* substituted (4-FBBA, **81**) was used to prove the importance of the boronic acid's at *ortho*-position and a substituted benzaldehyde with an *ortho*-potassium

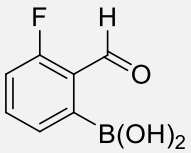
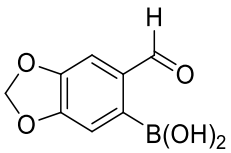
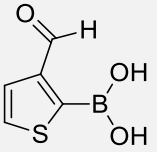
trifluoroborate (**82**) was used to show the importance of the vacant *p* orbital centered on boron. As expected, both species were unable to yield the desired imine in aqueous media as shown in Table **5**.

Table **5** – Boronated aldehydes reaction with 1-butylamine

Aldehyde/Ketone	Conversion of imine formed	Aldehyde/Ketone	Conversion of imine formed
 **81**	0%	 **82**	0%

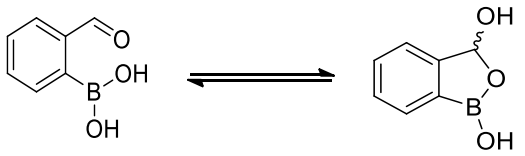
Aiming at improving the formation of the iminoboronate in aqueous media, several reaction parameters were evaluated, namely the reagent stoichiometry and dilution, buffer concentration and reaction time. However, all modifications had little effect on the iminoboronate formation. Apart from the reactions with unfunctionalized 2-FBBA, commercially available formylbenzeneboronic acids with different substituents (**83**-**85**) were also evaluated, yet, none significantly improved the conversion obtained before while using 2-FBBA (Table **6**).

Table **6** – 2-Formyl boronic acid derivatives' reaction with 1-butylamine

Aldehyde/Ketone	Conversion of imine formed	Aldehyde/Ketone	Conversion of imine formed	Aldehyde/Ketone	Conversion of imine formed
 **83**	18%	 **84**	53%	 **85**	0%

Based on the results obtained, a different approach was pursued to improve the imine formation with 2-FBBA, exploring the increase of the boronic acid electrophilicity/Lewis acidity upon complexation with cathecol, *p*-nitrocathecol and glucose as suggested in organic solvents by Ansyln *et al.*. However, in our study, the addition of these vicinal diols led to a considerable decrease of the imine formation. This fact may be related with the solvent insertion that disabled the possibility of N-B dative bond formation, by occupying more effectively the vacant *p* orbital of the boron atom.

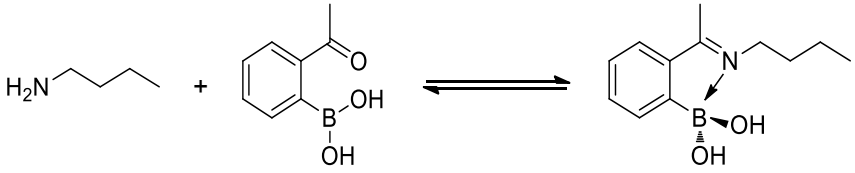
In the course or these studies with 2-FBBA, a competing reaction enhanced by the pH increasing was observed (Scheme **31**).[261] This tautomeric cyclization between the aldehyde and boronic acid was observable by ^{1}H-NMR, which led to a peak disappearance, related with the formyl proton, and the appearance of a new one at ~6.0 ppm, associated with the proton in the tertiary carbon in compound **86**. Thus, these results suggested that in this cyclized form (**86**), 2-FBBA becomes unable to react with 1-butylamine and this is probably the reason why throughout the optimization studies the yields of iminoboronates were never improved. After thorough NMR studies with 2-FBBA and 1-butylamine under acidic, neutral and basic pHs, it was possible to conclude that high pHs drive this equilibrium towards the unreactive compound **86** and acidic conditions protonate the amino group of 1-butylamine hampering this way the imine formation. Therefore, this dilemma couldn't be solved by tuning the reaction.



72 **86**

Scheme **31** – Tautomeric equilibrium of 2-FBBA

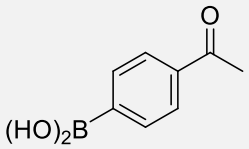
In order to reduce the impact of the competing reaction that leads to tautomeric compound **86**, the 2-acetylbenzeneboronic acid (2-ABBA, **88**) was tested as a substitute for 2-FBBA. It was hypothesized that the steric hindrance imposed by the methyl group could block the "reduction" of the carbonyl (Scheme **32**). Very gratifyingly, using 2-ABBA, the iminoboronate (**89**) was readily obtained in 88% conversion, either in neat water as in phosphate buffer solution (50 mM; pH 8.02). As expected, the *para*-isomer (4-ABBA, **90**), which is unable to establish the same envisioned N-B interaction was incapable to deliver the imine. Hence, compound **88** was selected as lead scaffold to develop protein modification of lysine residues.



87 **88** **89**

Scheme **32** – Reaction between 2-ABBA and 1-butylamine

Table **7** – 4-Acetylbenzeneboronic acid reaction with 1-butylamine

Aldehyde/Ketone	Conversion of imine formed
 **90**	0%

Once identified, 2-ABBA (**88**) was evaluated in these different studies involving the iminoboronate formation: kinetic evaluation and pH range assessment.

Considering the results obtained with the kinetic study, a high reaction rate was observed and the iminoboronate was already formed in 81% in deuterated water when the first ^{1}H-NMR was performed (after 15 minutes of reactants addition to the NMR tube). The iminoboronate's stability was also evaluated during this study, which demonstrated the construct's stability in water for up to 24 h (Figure **5**).

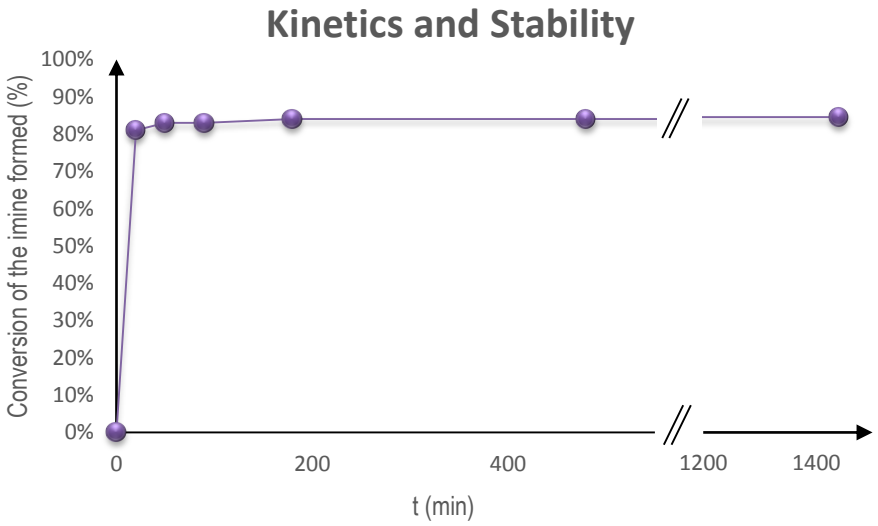


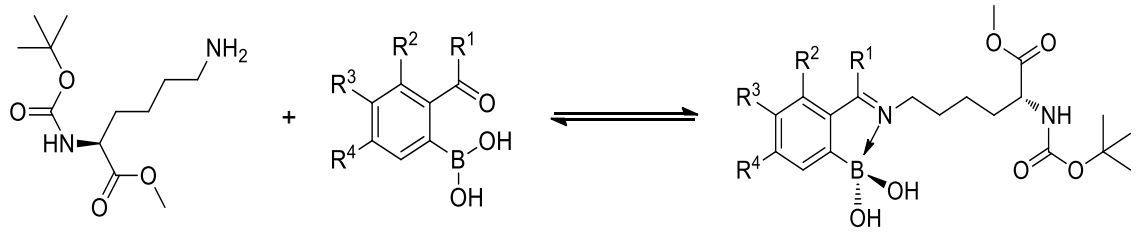
Figure **5** – Kinetics and stability of reaction between 2-ABBA and 1-butylamine

The formation of the iminoboronate **89** was also evaluated at pHs ranging from 6.0 up to 9.2 (Table **8**). This study showed a remarkable stability of the imine over 7 days under such conditions (room temperature and phosphate buffer solution, 50 mM).

Table **8** – Conversion from the reaction of 1-butylamine and 2-ABBA at different pHs

pH	*1 day*	*7 days*
6.0	67%	67%
7.0	79%	80%
8.0	84%	88%
9.2	87%	87%

Once the reaction conditions for the imine formation with 2-ABBA and 1-butylamine were well-established, we synthesized an *α*-protected lysine (**91**, Boc-Lys-OCH_3) which was then reacted with different 2-carbonyl boronic acids to determine the feasibility of this methodology when using more complex amines. Very gratifyingly, as shown in Table **9**, the reaction proceeded similarly to what was observed when using the model 1-butylamine, and again the 2-ABBA (**88**) proved to be the most efficient reagent yielding the iminoboronate in 71%.



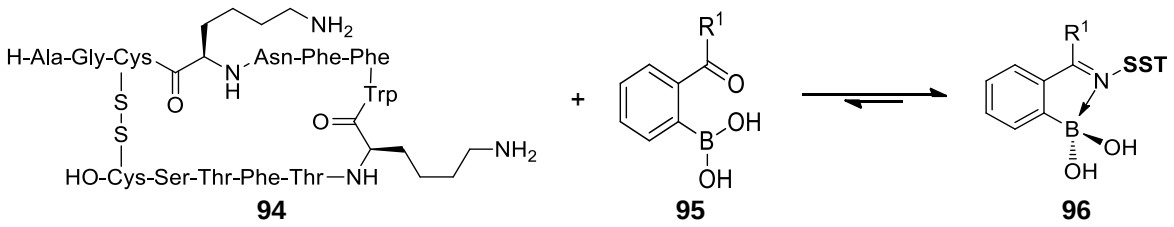
Scheme **33** – Reaction between 2-ABBA and Boc-Lys-OCH_3

Table **9** – Conversions from 2-carbonylbenzeneboronic acid derivatives' reaction with Boc-Lys-OCH_3

Compound	**-R^1**	**-R^2**	**-R^3**	**-R^4**	**Conversion**
2-FBBA, 72	-H	-H	-H	-H	61%
83	-H	-F	-H	-H	33%
84	-H	-H	-OCH_2O-	-OCH_2O-	56%
2-ABBA, 88	-CH_3	-H	-H	-H	71%

Taking these results in consideration, the functionalization of more complex biomolecules was then pursued. Somatostatin (SST) is a short-life hormonal neuropeptide, which inhibits the secretion of various hormones including the growth hormone. In tumor cells, membrane receptors for somatostatin are over-expressed, making it an excellent candidate to append and deliver cytotoxic agents.[262]

The 14 amino acid active form of this peptide was employed as a target, which presents two lysine residues in its sequence. SST-14 reacted with 2-ABBA (50 mM) for five minutes, in ammonium bicarbonate buffer (10 mM, pH 7.8) and the reaction was analysed by ESI-MS (Scheme **34** and Table **10**). Gratifyingly, the conjugates with one and two modifications were readily obtained with almost complete conversion of the peptide. Similarly, conjugation between 2-FFBA and SST-14 was evaluated, affording products with one, two and three modifications (*N*-terminal also modified). Once confirmed the successful formation of the constructs, MS2 analysis of the conjugate with three modifications obtained with 2-FBBA (m/z 1996.8) was performed and lead to the identification of a construct with one less appended boronic acid.



Scheme **34** – Reaction between 2-ABBA and SST

Table **10** – ESI-MS results from reaction 2-FBBA/2-ABBA with SST

Compound	-R^1	+1 BA (m/z)	+2 BA (m/z)	+3 BA (m/z)
2-FBBA, 72	-H	1750.7 ($-H_2O$)	1863.8 ($-2H_2O$)	1996.8 ($-2H_2O$)
2-ABBA, 88	$-CH_3$	1781.8	1927.8	-

Following the successful modification of somatostatin, the reaction was then attempted with a native protein under naturing conditions. Lysozyme (Lyz) is one of the most well characterized proteins with a single amino acid chain of 129 residues (six of which are lysines) and a molecular weight of 14.5 kDa. This enzyme is a glycoside hydrolase and it is known for reacting with peptidoglycans present in bacterial cells, which is why it has been successfully applied as a biopharmaceutical.[163,263]

As for the previous example, modification of Lyz with 2-ABBA took place smoothly at room temperature in ammonium acetate buffer (20 mM, pH 7.0), rendering up to six modifications evaluated with FTICR-MS (Figure **6**).

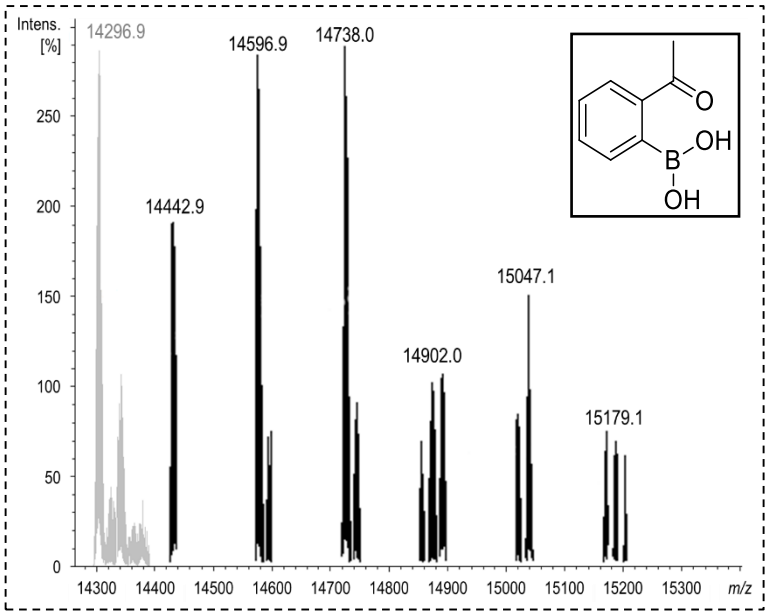


Figure **6** – Overlaid deconvoluted ESI-FTICR-MS spectra. **Grey** - unmodified Lyz; **Black** – Reaction between Lyz (10.0 μM) and 20.0 mM of 2-ABBA in ammonium acetate buffer (20.0 mM, pH 7.0)

Since lysozyme is rather stable and readily available,[264] it was used as a model protein for further studies, namely for the pH range assessment, the reaction's kinetic evaluation with 2-ABBA and 2-FBBA and also in an attempt to isolate the conjugates.

Remarkably, as it was true for the amine's alkylic model, the reaction is fairly tolerant to pH and stable through time. Successful modifications were obtained in neat water or in ammonium acetate buffer (20 mM) at pHs between 5.0 and 9.0, as well as insignificant changes were detected in the mass spectra from one up to four hs of reaction (under neutral pH).

Moreover, using the optimized conditions, lysozyme was reacted with 10 mM of 3-fluoro-2-formylphenylboronic acid (**83**), 2-formyl-4,5-methylenedioxyphenylboronic acid (**84**) and 3-formyl-2-thienylboronic acid (**85**) in ammonium acetate buffer (20 mM, pH 7.0) at room temperature (Figure **7**). Though quantitative measurements can not be directly accessed from the mass spectra, the herein reported results are in line with the ones obtained with the model 1-butylamine. The later boronic acid failed to react while the former two readily led to iminoboronate species, demonstrating the system tolerance towards different functionalities on the aromatic moiety.

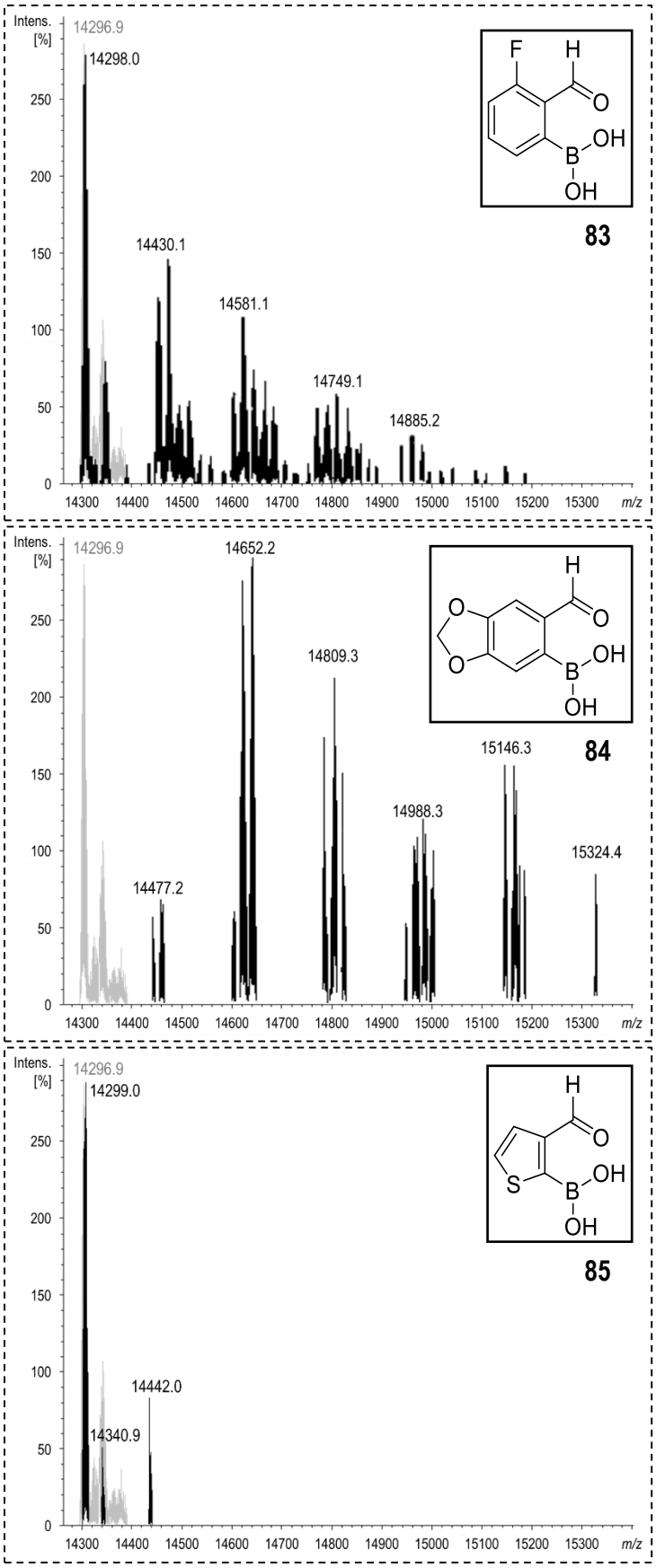


Figure **7** – Overlaid deconvoluted ESI-FTICR-MS spectra. **Grey** - unmodified Lyz; **Black** – Reaction between Lyz (10.0 μM) and 10.0 mM of compounds **83** or **84** or **85** in ammonium acetate buffer (20.0 mM, pH 7.0)

Despite the several attempts of purifying the conjugates obtained from 2-ABBA and Lyz were performed, its isolation was not achieved. Techniques such as dialysis (with D-tube™ dialyzers or Amicon® ultra diafilters), size exclusion gravity columns (with Thermo Scientific™ Pierce™ dextran desalting columns) and centrifugal filtration (with Vivaspin® 2 centrifugal concentrator) were unsuccessfully executed.

Finally, after the unproductive attempts of isolating the iminoboronate conjugates with lysozyme, other model proteins were targets of this conjugation method to show the vast applicability of this procedure. With the optimized conditions obtained for lysozyme, cytochrome c, ribonuclease A and myoglobin were also fully converted with 2-FBBA (**72**) in less than 5 min after addition of this boronic acid at room temperature, as depicted in Figure **8**, Figure **9** and Figure **10**, respectively.

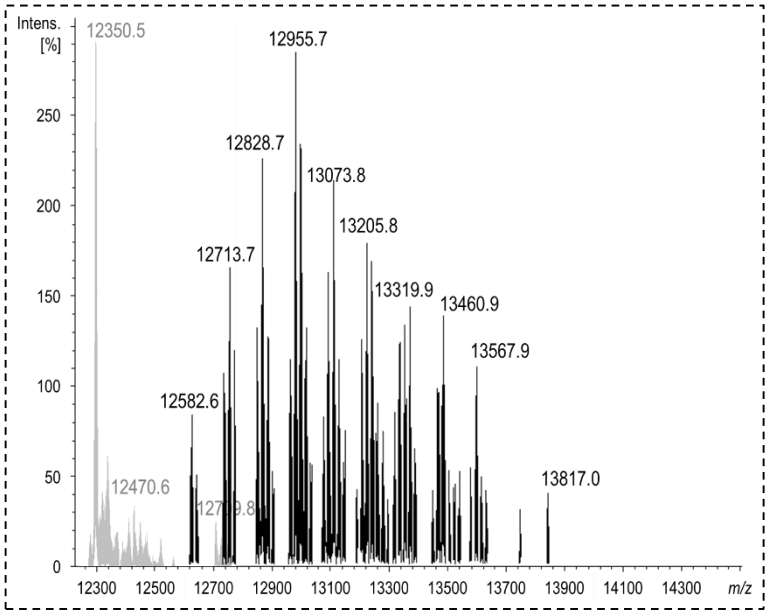


Figure **8** – Overlaid deconvoluted ESI-FTICR-MS spectra. **Grey** - unmodified cytochrome C; **Black** – Reaction between cytochrome C (10.0 μM) and 10.0 mM of 2-FBBA in ammonium acetate buffer (20.0 mM, pH 7.0)

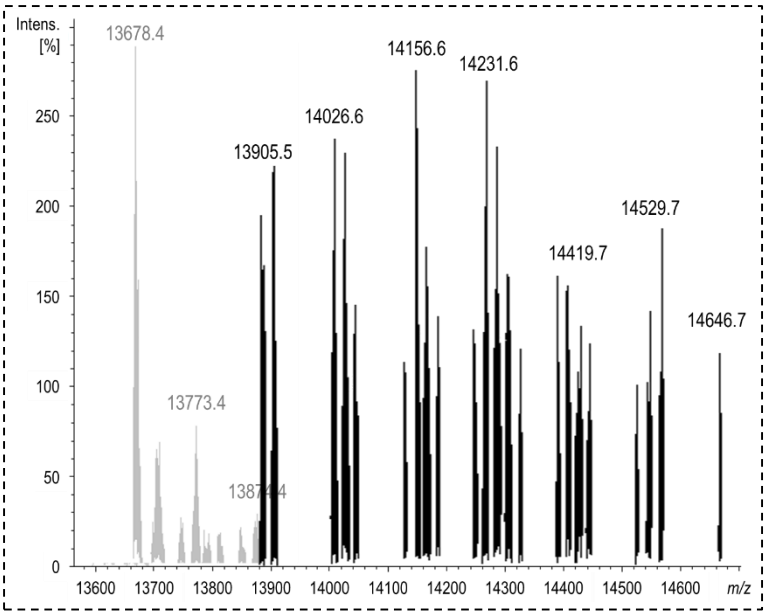


Figure **9** – Overlaid deconvoluted ESI-FTICR-MS spectra. **Grey** - unmodified ribonuclease A; **Black** – Reaction between ribonuclease A (10.0 μM) and 10.0 mM of 2-FBBA in ammonium acetate buffer (20.0 mM, pH 7.0)

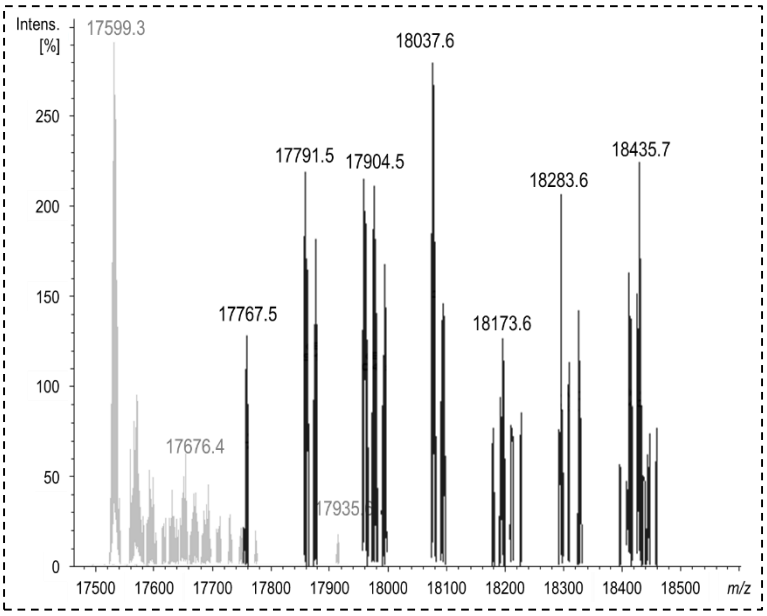


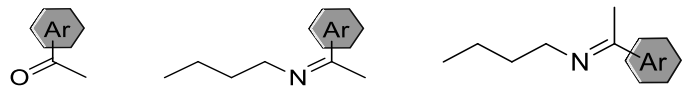
Figure **10** – Overlaid deconvoluted ESI-FTICR-MS spectra. **Grey** - unmodified myoglobin; **Black** – Reaction between myoglobin (10.0 μM) and 10.0 mM of 2-FBBA in ammonium acetate buffer (20.0 mM, pH 7.0)

Hence, based on all the reported experimental data, it is fair to state that a new conjugation system selective for primary amines has been developed. In order to further understand the importance of the boronic acid in this conjugation system, the reaction mechanism was studied using DFT calculations.

II.3. Computational Studies

Taking into account the experimental results discussed in **II.2 Exploratory Results and Optimization**, it is comprehensible that further information regarding the mechanism of iminoboronate formation and the N-B interaction should be of great value and an excellent complement to the previously discussed results. With that purpose, the well-known reaction of imine formation was studied by means of DFT calculations, using 1-butylamine as the amine model counterpart and different acetyl compounds to support the claim of a remarkable stability of the iminoboronate towards hydrolysis. First, acetophenone was used showing a well-known two step mechanism of hemiaminal formation and subsequent dehydration to generate the imine. Secondly, the same mechanism was applied for a boronic acid derivative, 4-ABBA, showing that the relative position of this chemical group makes a difference in terms of aiding imine formation, as well as the final product stabilization. Finally, 2-ABBA was used as a carbonyl counterpart to verify the role of the boronic acid in the *ortho* position to the acetyl group. Different mechanisms are purposed for this last reaction and all will be discussed in detail.

It is important to highlight that for all mechanisms studied, there are two representations of each compound corresponding to diferent *cis* and *trans* imine stereoisomers. While the acetyl reagent (compound **97,** Figure **11**) does not present any isomer about the double bond, in the imine double bond, butylamine acts as a third substituent in this double bond, having an assigned priority, defined by a set of priority rules made by Cahn-Ingold-Prelog. Thus, two diastereoisomers are formed and this will have a crucial importance, in terms of possible N-B interactions between the boronic acid in the aryl group (Ar) and the nitrogen atom from 1-butylamine. When the aryl and the butyl group are on the same side of the double bond, the bond is assigned the configuration *Z*, like demonstrated in Figure **11** with compound **98**. When these higher priority groups are faced on opposite sides of the double bond, this linkage is assigned with the configuration *E*, as shown with compound **99** (Figure **11**).



97 **98** **99**

Figure **11** – Acetyl reagent and different diastereoisomers for Imine product

II.3.1. Acetophenone and 1-Butylamine

As aforementioned and as it was done with the presented experimental data, DFT calculations were performed focusing on the reaction between an aboronated carbonyl compound, acetophenone (**80**) and 1-butylamine (**87**). Within the objective of this thesis, these results could be later compared with the ones obtained for the iminoboronate formation.

This textbook mechanism of imine formation is based on two fundamental steps, which are two proton transfers from the nitrogen of an amine to the oxygen of a carbonyl compound driven by a nucleophilic attack from the amine to the electrophilic carbon of the carbonyl group. In opposition to iminoboronates, after imine formation, there is no possible stabilization in any of the isomers. Both isomers of this reaction are represented in Figure **12** and Figure **13** highlighting their similar energy.

The mechanism for each isomer, starts with **A,** where the reactants (1-butylamine (**87**) and acetophenone (**80**)) interact in the presence of an additional water molecule that facilitates the proton transfers between these species. In the first step, from **A** to **B**, there is an approach made by the nitrogen to the carbonyl's *C*-atom (from $d_{\mathbf{A}}$N–C = 3.73 Å to $d_{\mathbf{B}}$N–C = 1.58 Å), which leads to a simultaneous protonation of the oxygen atom, forming the expected hemiaminal **B**. This proton transfer is aided by a neighbour water molecule that exchanges one N-H proton from the amine to the water molecule. Simultaneously, one of the water O-H protons is transferred to the *O*-atom of the carbonyl group. In intermediate **B**, a new C-N bond is formed, as attested by the distance between these atoms ($d_{\mathbf{B}}$N–C = 1.43 Å).

From **B** to **C** there is a rearrangement of the geometry of the pair hemiaminal-water and of the corresponding *H*-bond network, which is required in order for the same water molecule to transfer another N-H proton from the now secondary amine to the newly formed secondary alcohol. In this following step (from **D** to **E**) a second molecule of water is formed, while a C-O bond is broken ($d_{\mathbf{D}}$C–O = 3.35 Å) and the C-N imine **D** double bond formed ($d_{\mathbf{D}}$N–C = 1.28 Å).

Considering the thermodynamics of the process, these results highlight an unfavourable reaction pointing out a reversible character generally associated with a well-known tendency of imines to suffer hydrolysis. For instance, the last *O*-protonation step is highly unfavourable, even though a solvent molecule was considered to assist the process, $\mathbf{TS_{C\text{-}D}}$ still presents 30-31 kcal/mol above the reactants (**A**). Moreover, this step is slightly endo-energetic (ΔE = 3-5 kcal/mol), indicating that there is a stability loss between the hemiaminal and the resulting imine. Additionally, an overall energy balance of 3-5 kcal/mol and an energy of activation for this reaction of 35-36 kcal/mol also support the claims of a reversible and unfavourable reaction.

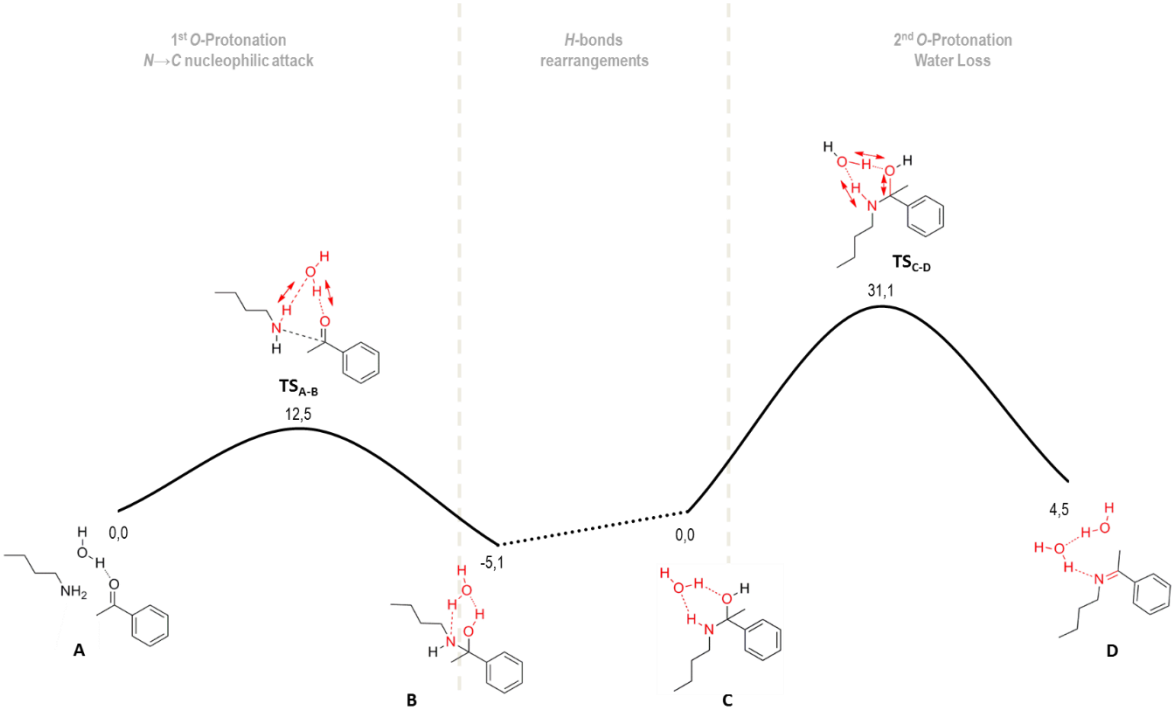


Figure **12** – Energy profile (kcal/mol) calculated (PBE0/6-31G**) for the formation of the *Z*-isomer of *N*-butylmethylphenylimine from 1-butylamine (**87**) and acetophenone (**80**). Relevant changes are highlighted in red.

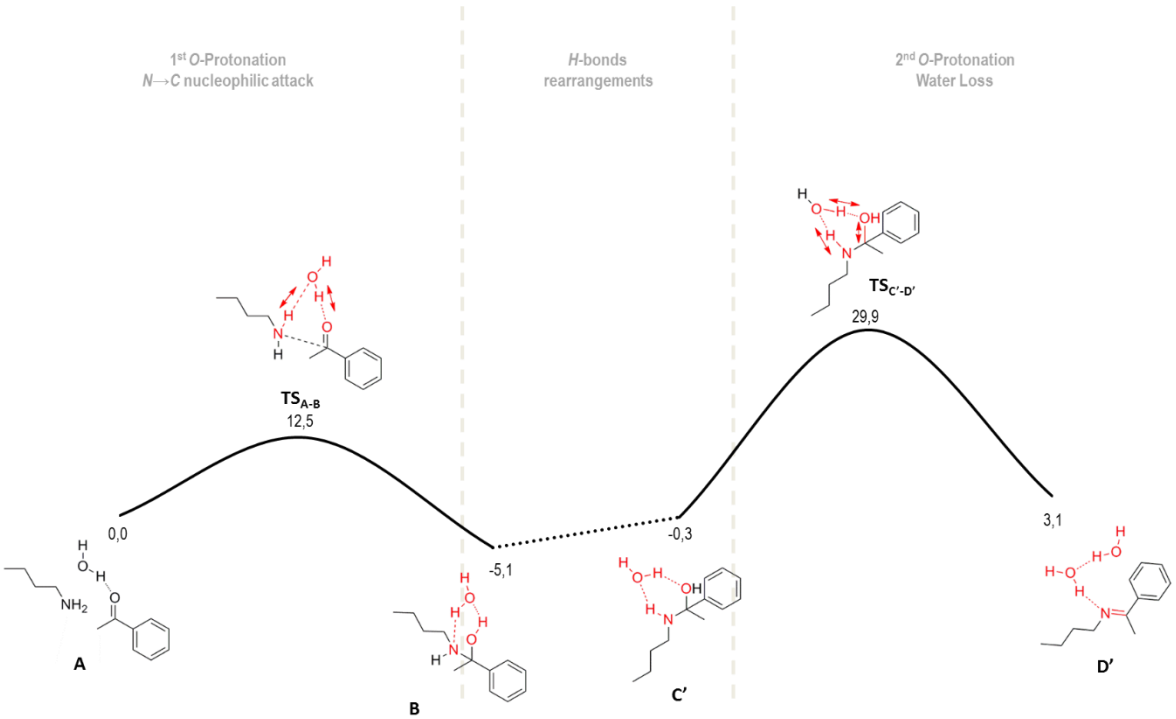


Figure **13** – Energy profile (kcal/mol) calculated (PBE0/6-31G**) for the formation of the *E*-isomer of the *N*-butylmethylphenylimine from 1-butylamine (**87**) and acetophenone (**80**). Relevant changes are highlighted in red.

Isomers *Z* and *E* only vary on 1-2 kcal/mol *per* step and therefore, all that it has been mention from **A**-**D** it is also true for **A**-**D'**.

In conclusion, this far, all theoretical data is coherent with the preliminary experimental results obtained upon reaction of 1-butylamine (**87**) and acetophenone (**80**) in water, at room temperature, with a detected conversion of 0% (Table **4**).

II.3.2. 4-Acetylbenzeneboronic acid and 1-Butylamine

Furthermore, in the same line of thought as the one applied with the experimental data, the reaction mechanism between 1-butylamine (**87**) and 4-ABBA (**90**) was also evaluated by means of DFT calculation, and as shown in Figure **14** and Figure **15**, no significant changes were detected when comparing with the mechanism of 1-butylamine and acetophenone.

This mechanism elucidation serves the purpose of stressing the importance of the position of the boronic acid within the aromatic ring for stabilizing the newly-formed imine. Considering the experimental and theoretical studies, solubility issues are unlikely responsible for the differences in reactivity of 2-ABBA and butylamine when compared with acetophenone.

As a general remark, the steps depicted in Figure **14** and Figure **15** follow exactly the same routes as the previous mechanism, so they will not be discussed in detail. Summarizing, this mechanism also describes a thermodynamic unfavourable process and stresses the same reversible character. Similar energies as the ones from the last mechanism were obtained, like the slightly endo-energetic last step (ΔE = 3-4 kcal/mol) and the energy activation calculated for this reaction of 34-35 kcal/mol. Again, both mechanisms for isomers *Z*- and *E*- also have little variations.

In conclusion, these theoretical studies support the experimental results obtained with the reaction of 1-butylamine (**87**) and 4-ABBA (**90**) in water, at room temperature, where the imine was not formed (Table **7**).

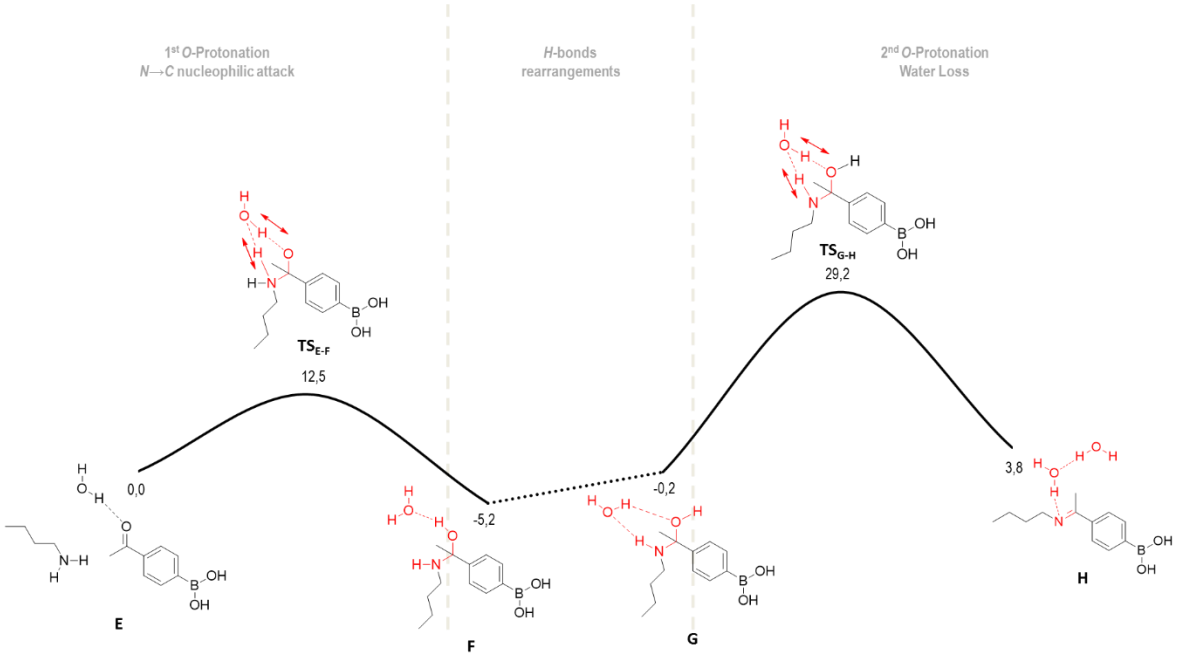


Figure **14** – Energy profile (kcal/mol) calculated (PBE0/6-31G**) for the formation of the *Z*-isomer of the iminoboronate obtained from 1-butylamine (**87**) and 4-ABBA (**90**). Relevant changes are highlighted in red.

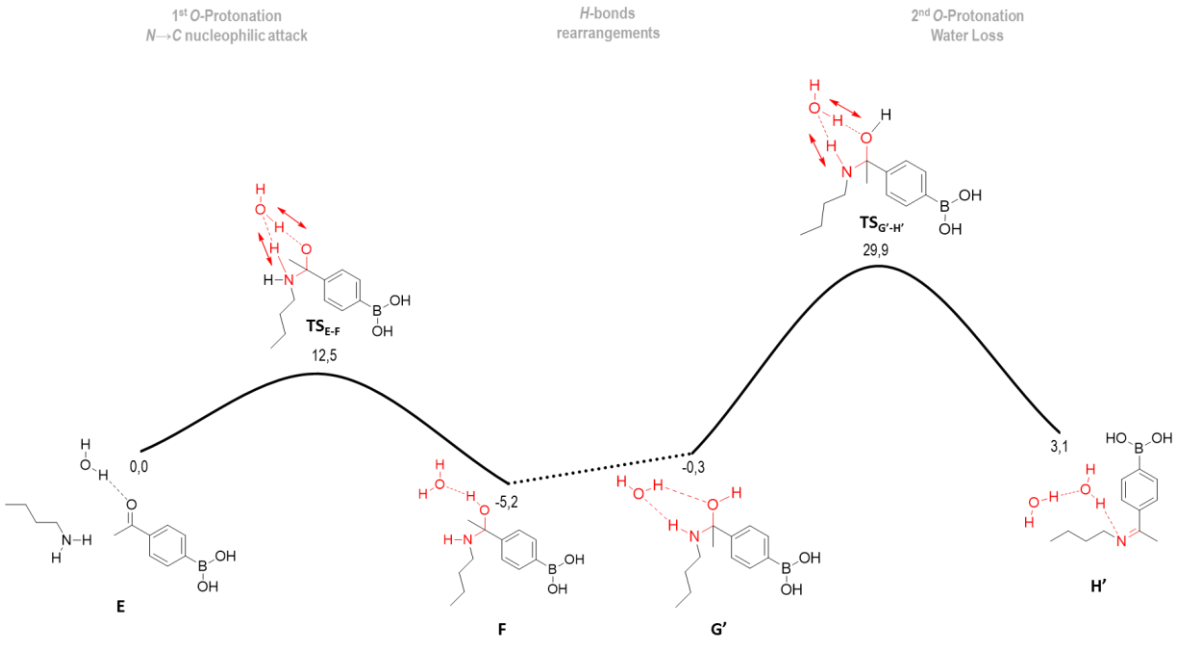


Figure **15** – Energy profile (kcal/mol) calculated (PBE0/6-31G**) for the formation of the *E*-isomer of the iminoboronate obtained from 1-butylamine (**87**) and 4-ABBA (**90**). Relevant changes are highlighted in red.

II.3.3. 2-Acetylbenzeneboronic acid and 1-Butylamine

Finally, the mechanism of the reaction between 1-butylamine (**87**) and 2-ABBA (**88**) was studied by means of DFT calculations. However, in this case, different interactions between the reactants led to the development of three different hypothetic mechanisms.

In both hypothetic mechanisms 1 and 2, imine formation is the last step and the boronic acid's role is the most noticeable difference between the two mechanisms. In the first case, boron acts as a Lewis acid forming a zwitterionic species between a hydroxylated benzoxaborole and a protonated secondary amine (**TS$_{J-K}$**, Figure **17**), although boron is not directly involved in the protonation step. In the second mechanism, boron acts as a Brønsted acid, directly protonating a hydroxyl function of the newly formed secondary alcohol (**TS$_{Q-R}$**, Figure **20**), which is known to be a highly unfavourable process. Regarding the hypothetic mechanism 3, the boronic acid also acts as a Lewis acid, but the iminium is formed in early steps of the mechanism, leading to a zwitterionic species where a free boronate is its counterpart (**TS$_{T'-U'}$**, Figure **24**). Also, it is worth highlighting that in any of the studied mechanisms there is always a final step of N-B coordination in all *E*-isomers, a bond that due to steric hindrance can not be formed in the *Z*-isomers.

Hypothetic mechanism 3 was found to most effectively explain the observed results and for that reason, only this mechanism will be compared with the previous ones.

II.3.3.1. Hypothetic Mechanism 1

Regarding the first hypothetic mechanism of the reaction between 1-butylamine (**87**) and 2-ABBA (**88**), the *ortho*-boronic acid relatively to the carbonyl function acts as a Lewis acid. This leads to the formation of an intermediary benzoxaborole, which upon protonation regenerates the boronic acid functionality, as represented in Figure **16** and Figure **17**. In this case, the mechanisms representing *Z*- and *E*-isomers formation present significant energetic differences and will be discussed in detail.

The formation of the *Z*-isomer of iminoboronate **N** involves four consecutive steps, starting from the reagents **I**, 1-butylamine (**87**), 2-ABBA (**88**) and an additional water molecule to mediate proton transfers. In the first step, from **I** to **J**, there is a coordination of the *O*-atom of the carbonyl group to the boron ($d_{TS_{I-J}}$B-O = 1.87 Å), while the nitrogen attacks the electrophilic carbon from the carbonyl group ($d_{TS_{I-J}}$N-C = 2.64 Å). This step leads to a zwitterionic species between a protonated secondary amine (d_JN-C = 1.6 Å) and a hydroxylated benzoxaborole (d_JB-O = 1.54 Å) and not the usual hemiaminal as one might expect.

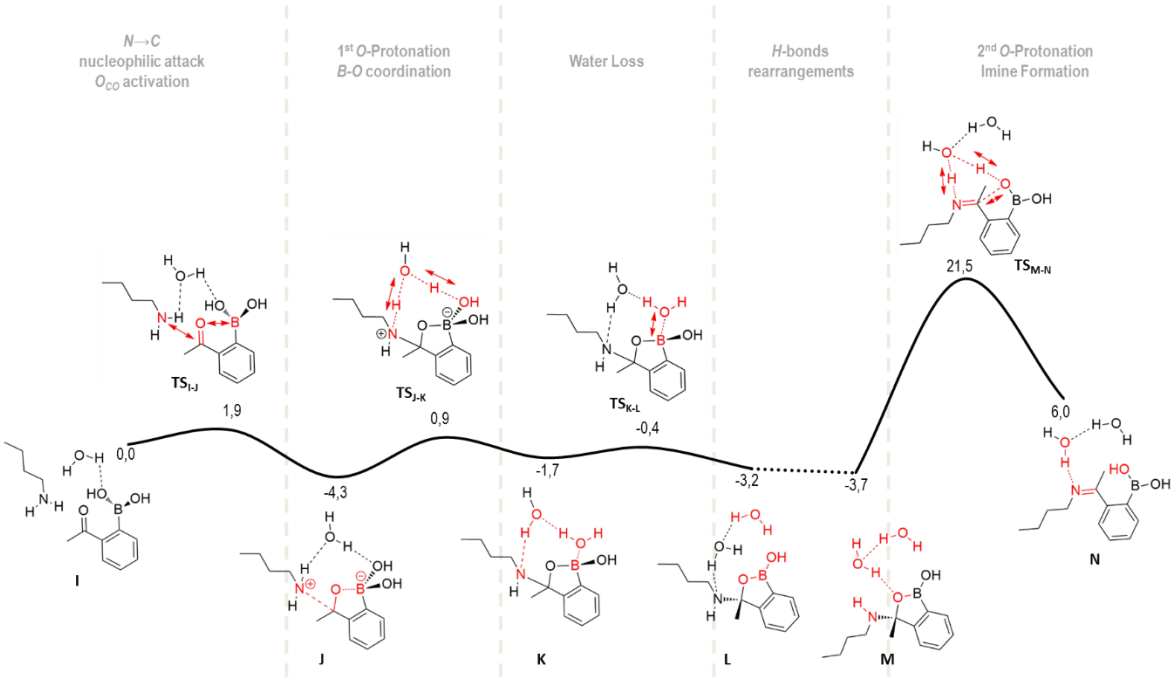


Figure **16** – Energy profile (kcal/mol) calculated (PBE0/6-31G**) for the formation of the *Z*-isomer of the iminoboronate obtained from 1-butylamine (**87**) and 2-ABBA (**88**) – Mechanism 1. Relevant changes are highlighted in red.

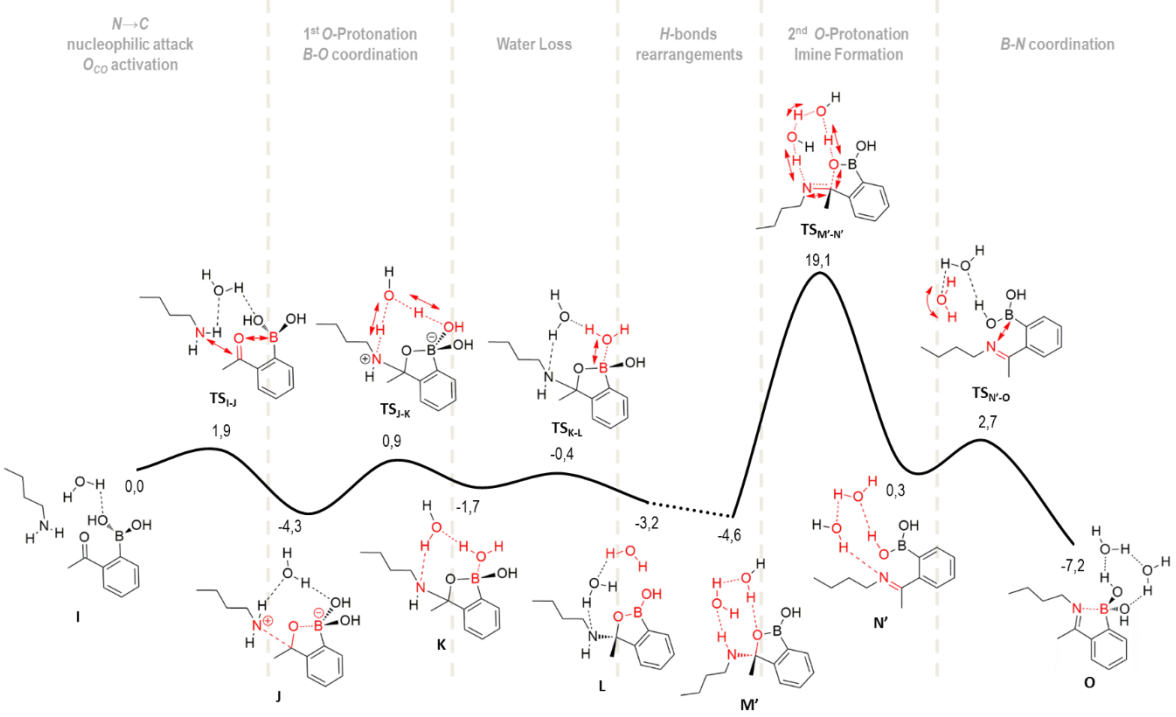


Figure **17** – Energy profile (kcal/mol) calculated (PBE0/6-31G**) for the formation of the *E*-isomer of iminoboronate (**89**) from 1-butylamine (**87**) and 2-ABBA (**88**) – Mechanism 1. Relevant changes are highlighted in red.

This step is followed by a process of proton transfer aided by the neighbour water molecule, from the N-H of the protonated amine to one hydroxyl groups of the cyclic boronate. This step leads to a neutral species where the water molecule remains coordinated to the boron atom (d_{K}B-O = 1.64 Å). In the consecutive step a water molecule is loss and the oxygen and the boron atoms are incoordinated (d_{L}B-O = 3.26 Å). The optimized geometries of tetrahedral coordinated benzoxaborole in **K** and the trigonal uncoordinated benzoxaborole in **L** are depicted in Figure **18**.

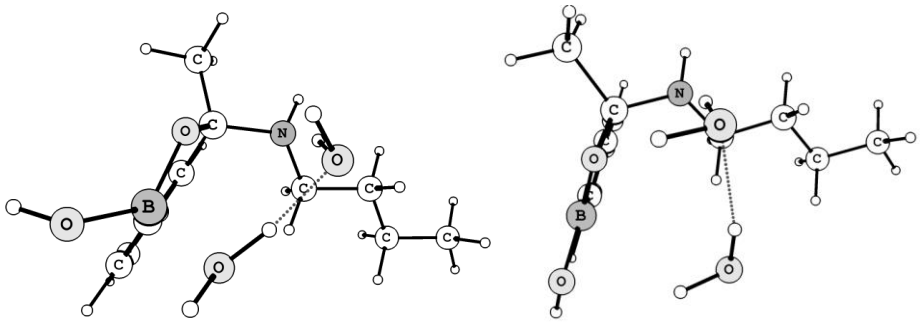


Figure **18** – Illustrations of the optimized geometry (PBE0/6-31G**) of **K** (left) and **L** (right) to highlight boron's coordination

From **L** to **M** there is the already mentioned *H*-bond rearrangement using the same water molecule to transfer another proton. This transfer is the fourth and last step of the iminoboronate *Z*-isomer's mechanism 1 (from **M** to **N**) and it is assisted by the neighbour water molecule to move another N-H proton from the newly formed secondary amine to the cyclic *O*-atom of the benzoxaborole structure. This procedure finally leads to imine formation (d_{N}N-C = 1.28 Å), by cleavage of the C-O bond and subsequent regeneration of the boronic acid (d_{N}B–O = 1.36 Å). Overall, the formation of the iminoboronate *Z*-isomer in mechanism 1 is exo-energetic, as product **N** is 6.0 kcal/mol less stable than the reactants in **I**.

Due to the rigidity associated with a double bond, no subsequent stabilization can occur in the *Z*-isomer, justifying the product instability when compared to the reactants. In the case of iminoboronate *E*-isomer's mechanism 1 (Figure **17**), once the imine is formed due to a favourable internal geometry adjustment, the *N*-atom and the boron are close enough to establish a N-B bond (d_{O}N-B= 1.71 Å) greatly enhancing the stability of the product **O**. In fact, the last step is strongly exo-energetic (ΔE = –7.5 kcal/mol), indicating the stability gain resulting from the formation of the N-B bond. This also reflects in an overall thermodynamic favourable energy balance of –7.2 kcal/mol.

Despite being a thermodynamic favourable process , the energy activation calculated for the reaction (23.7 kcal/mol) that corresponds to the highest barrier in the mechanism (from **M** to **N**) is similar to the

previous species evaluated by DFT calculations. Therefore, this mechanism does not support the experimental results. In fact, the transition state for the second *O*-protonation, $\mathbf{TS_{M'-N'}}$, although assisted by a solvent molecule, is 19-21 kcal/mol less stable than the reactants **I**. Such high energy for a reaction that occurs smoothly at room temperature led us to investigate other mechanisms, aiming to unveil new roles of the boronic acid to stabilize the second *O*-protonation.

Before moving on to the next hypothetic mechanism though, it is important to summarize the role that the boronic acid took in this mechanism 1, since it is considered to be the driving force of this reaction and also the reason for stabilization of the final product. Thus, in the first step, boronic acid establishes a B-O bond with the oxygen from the carbonyl group in order to activate this group towards a nucleophilic attack from the amine's *N*-atom. Then it forms a benzoxaborole group (coordinated or not with water) that will be present until the second *O*-protonation that leads to imine formation. As expected also in the *E*-isomer formation, the establishment of the N-B dative bond that stabilizes the final product.

II.3.3.2. Hypothetic Mechanism 2

Aiming at discovering a reaction mechanism between 1-butylamine (**87**) with 2-ABBA (**88**) that supports the results obtained experimentally, an alternative mechanism was studied by means of DFT calculations. In Figure **19** is presented the calculated mechanism for the *Z*-isomer and Figure **20** for the *E*-isomer.

This mechanism for iminoboronate formation presents two major differences from the ones presented before. First, it leads to the expected hemiaminal, though it requires overcoming higher energy barriers (8.5 kcal/mol) to achieve the intermediary **R**. Also in contrast with the previous mechanism, where water loss is driven by an uncoordination between the boron atom and the water molecule (from **K** to **L**), in hypothetic mechanism 2, the water loss is driven by the direct protonation from one of the hydroxyl groups of the boronic acid to the hydroxyl group of the newly formed secondary alcohol (from **S** to **M''**). Therefore, in this mechanism the boronic acid plays a role of a Brønsted acid, which is not a thermodynamic favourable process, as confirmed by the energy requirements.

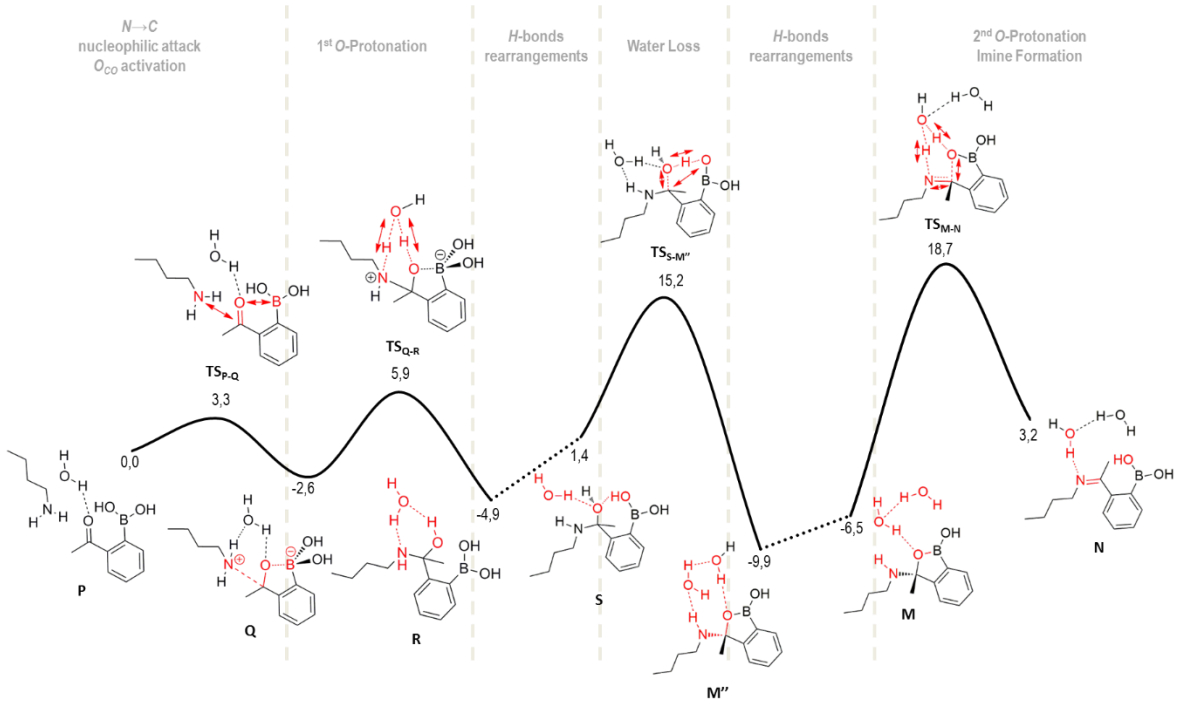


Figure **19** – Energy profile (kcal/mol) calculated (PBE0/6-31G**) for the formation of the *Z*-isomer of the iminoboronate obtained from 1-butylamine (**87**) and 2-ABBA (**88**) – Mechanism 2. Relevant changes are highlighted in red.

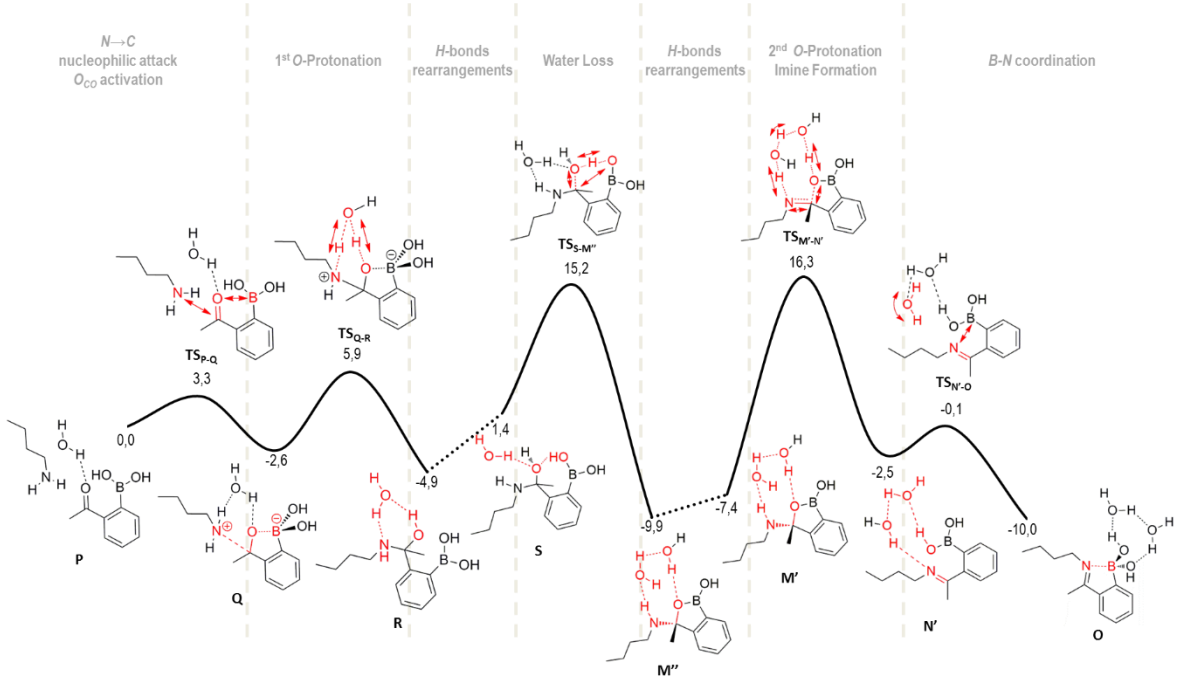


Figure **20** – Energy profile (kcal/mol) calculated (PBE0/6-31G**) for the formation of the *E*-isomer of iminoboronate (**89**) from 1-butylamine (**87**) and 2-ABBA (**88**) – Mechanism 2. Relevant changes are highlighted in red.

Hypothetic mechanism 2 involves four consecutive steps with an extra one of N-B coordination for the *E*-isomer as described before in mechanism 1. With either isomer, this mechanism starts with **P**, the reactants and an additional water molecule, which then through a nitrogen's nucleophilic attack to the electrophilic carbon of the carbonyl ($d_{\mathbf{Q}}$N–C = 1.59 Å) leads to a coordination of the oxygen to the boron atom ($d_{\mathbf{Q}}$B–O = 1.63 Å).

From **Q** to **R**, the aforementioned nucleophilic attack is completed with the formation of a new N-C bond ($d_{\mathbf{R}}$N–C = 1.48 Å) and simultaneous protonation of the oxygen atom from the previous carbonyl group, leading to hemiaminal **R**. As in the case of the other mentioned mechanisms, a neighbour water molecule assists the proton transfer from the amine group to the oxygen atom of the carbonyl group.

From **R** to **S** there is a rearrangement of the geometry of the hemiaminal-water pair and of the corresponding *H*-bond network, in a way that the proton from one of the hydroxyl groups of the boronic acid forms a strong *H*-bond with the newly formed secondary alcohol ($d_{\mathbf{S}}$BOH-O = 1.76 Å).

From intermediate, **S**, based on the close proximity between the hydrogen and the alcohol, the subsequent step is established by breaking the C-O bond and releasing a second water molecule. In the corresponding high energy transitory state, $\mathbf{TS_{S\text{-}M''}}$ (Figure **21**), the former C–O bond is practically cleaved ($d_{\mathbf{TS_{S\text{-}M''}}}$C–O = 2.34 Å) and comprises a boronic acid acting as a Brønsted acid. The boronic acid yields a proton to promote the release of a new molecule of water, instead of the normal path of proton transfer from the second proton of the amine. This last step has a high energy barrier showing a step strongly endo-energetic (ΔE = 13.8 kcal/mol) and indicating that this is not a suitable role for this organic group, as expected and commented in **II.1.2 Boronic Acids**. This step also leads to the formation of a benzoxaborole group mentioned in mechanism 1, **M''**, since the boronic acid is not stable when bearing an unprotonated hydroxyl group.

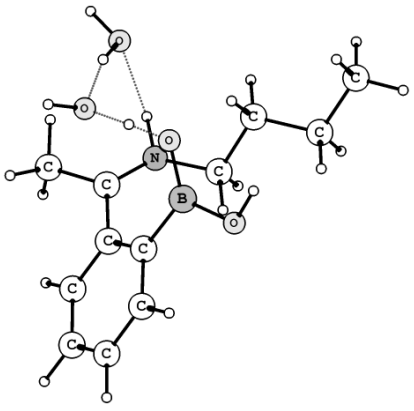


Figure **21** – Water loss promoted by boronic acid in the fourth step of hypothetic mechanism 2 – Optimized geometry (PBE0/6-31G**) of $\mathbf{TS_{S\text{-}M''}}$

From **M''** to **M** there is another rearrangement of the geometry and corresponding *H*-bond network, though in this case between the benzoxaborole-water pair, since with the previous step of water loss, the heminaminal no longer exists. This reorganization gives the proper geometry for the final step of this mechanism to occur, a second *O*-protonation. This last step, from **M** to **N**, is based on a proton transfer from the second proton of the nitrogen atom to the benzoxaborole group, which is assisted by the neighbour water molecular forming the imine and regenerating the boronic acid.

All the steps described so far are based on the iminoboronate *Z*-isomer's mechanism 2, though all descriptions are also applicable to the *E*-isomer with slight energetic changes from **M'** to **N'**, as the rearrangement of the geometry of the pair benzoxaborole-water and corresponding *H*-bond network is different to afford this different diastereoisomer.

Moreover, the *E*-isomer has an additional step of N-B coordination, which is mainly related with an internal geometry adjustment of a NC–C(phenyl) bond rotation that brings the imine's N-atom into closer proximity of the boron atom, allowing the formation of a new N-B bond (d_{o}N-B = 1.71 Å). This last step is once again, strongly exo-energetic (ΔE = –7.5 kcal/mol) representing a stability enhancement for this bond formation.

The overall energy balance of –10.0 kcal/mol represents a favourable thermodynamic process, though the high activation energy calculated for this reaction (26.2 – 28.3 kcal/mol) corresponding to the highest barrier along the path (from **M** to **N**) demonstrates the same drawback as in the last hypothetic mechanism. The protonation step of the cyclic oxygen from benzoxaborole is a high energy step that once more is not supported by the experimental data. Furthermore, in this hypothetic mechanism there is another high activation energy step to obtain the benzoxaborole, which is related with the unusual acidic nature of the boronic acid. Thus, the corresponding transitory state $\mathbf{TS_{S\text{-}M''}}$ is 15.2 kcal/mol less stable when compared to the reactants **P**. For these reasons, once again this hypothetic mechanism 2 is believed to be unsuitable to describe the smooth reaction between 1-butylamine and 2-ABBA that occurs at room temperature. Once more, other roles for the boronic acid were investigated.

As performed in the hypothetic mechanism 1, due to its importance the boronic acid's part in this mechanism will now be summarized before moving to the next hypothetic mechanism. As before, in the first step, the boronic acid forms a B-O bond with the O-atom from the carbonyl group to activate it towards the nucleophilic attack from the amine's *N*-atom. However, in the water loss step the boronic acid plays a direct role in *O*-protonation of the newly formed secondary alcohol and also one of its oxygens forms a C-O bond that did not exist previously. This is the major difference between this mechanism and the previous one. The other functions performed by this group are the same as in hypothetic mechanism 1 – benzoxaborole formation and stabilization of the final product (for the *E*-isomer).

II.3.3.3. Hypothetic Mechanism 3

Based on the aforementioned results a third hypothetic mechanism for the reaction between 1-butylamine (**87**) and 2-ABBA (**88**) was studied by means of DFT calculations, again for the *Z*- and *E*-isomer depicted in Figure **22** and Figure **24**, respectively.

Differently from all the calculated mechanisms presented until now, the diastereoisomers presented significant energy differences, whether for its final product as for some steps along the path. For that reason, both will be fully discussed with the exception of heminal formation and steps from **P** to **R** (which are the same described in hypothetic mechanism 2).

Considering the mechanisms presented in Figure **22** and Figure **24**, when going from **R** to **N'** (for *Z*-isomer) or from **R** to **O** (for *E*-isomer), these paths proceed through formation of an iminium species and a boronate group forming different zwitterionic intermediaries. Benzoxaborole group was not detected in these mechanisms calculated for both isomers.

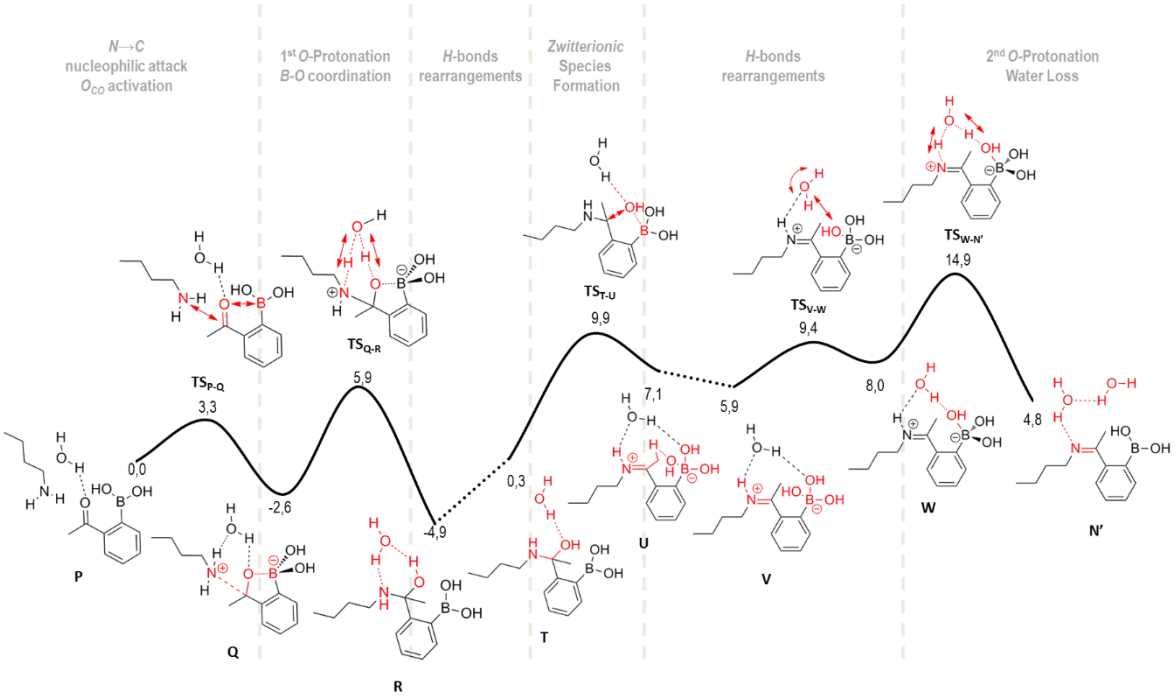


Figure **22** – Energy profile (kcal/mol) calculated (PBE0/6-31G**) for the formation of the *Z*-isomer of the iminoboronate obtained from 1-butylamine (**87**) and 2-ABBA (**88**) – Mechanism 3. Relevant changes are highlighted in red.

Regarding the mechanism calculated for the *Z*-isomer (Figure **22**), when going from **R** to **T** there is a rearrangement of the geometry of the hemiaminal-water pair and of the corresponding *H*-bond network. Then, in the following step, from **T** to **U**, there is cleavage of a C-O bond, transferring the newly formed hydroxyl group to the boron atom, in line with the true character of this mild Lewis acid. Here the resulting negative charge is formally drawn on the boron atom but, in fact, it is spread out on the three heteroatoms. In parallel with boronate formation there is also formation of an iminium ion, which then forms the first abovementioned zwitterionic species, **U**. The corresponding transition state, $\mathbf{TS_{T\text{-}U}}$, represents several characteristics that corroborate these claims, namely the former C–O bond practically cleaved ($d_{\mathbf{TS_{T\text{-}U}}}$C-O = 2.13 Å), the new B-O bond ($d_{\mathbf{TS_{T\text{-}U}}}$B–O = 1.55 Å) and the new C=N+ bond ($d_{\mathbf{TS_{T\text{-}U}}}$N–C = 1.32 Å) newly formed.

From **U** to **W** two reorganization of the *H*-bonds between the boronate-water-iminium triad occur, which lead to the release of a water molecule and the final iminoboronate in the *Z*-isomer form (from **W** to **N'**). This second *O*-protonation represents one of the major energetic differences between the mechanisms of both isomers, mainly due to a stereochemical arrangement around the C=N bond of $\mathbf{TS_{W\text{-}N'}}$ that prevents the proper organization for the water molecule to mediate a proton transfer, as depicted in Figure **23**. However this limitation might be overcome by using two water molecules (instead of one) that would facilitate this proton transfer, which is reasonable considering that water is the reaction's solvent.

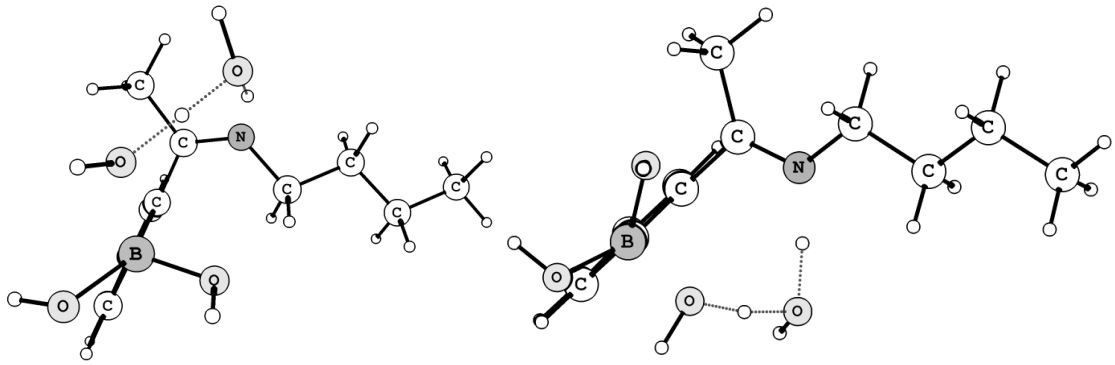


Figure **23** – Illustrations of the optimized geometry (PBE0/6-31G**) of $\mathbf{TS_{W\text{-}N'}}$ (left) and $\mathbf{TS_{U'\text{-}V'}}$ (right) to highlight steric hindrance

Regardless of that fact, the *Z*-isomer of the iminoboronate **N'** can not establish the N-B interation due to its stereochemical arrangement. The lack of this property leads to an intermediate 4.8 kcal/mol less stable than the initial reactants **P**, and hence 15 kcal/mol less stable than **O**. Thus, suggesting that *Z*-isomer of this iminoboronate corresponds to a thermodynamically unfavourable reaction and consequently, easily reversible.

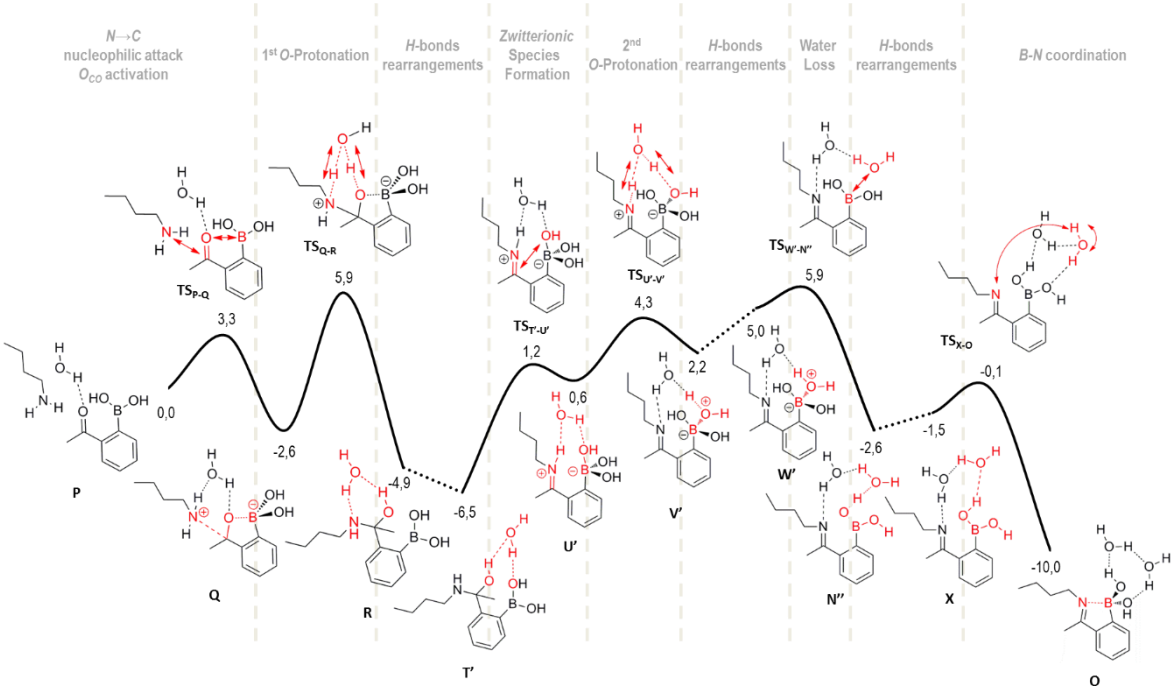


Figure **24** – Energy profile (kcal/mol) calculated (PBE0/6-31G**) for the formation of the *E*-isomer of iminoboronate (**89**) from 1-butylamine (**87**) and 2-ABBA (**88**) – Mechanism 3. Relevant changes are highlighted in red.

In the case of the *E*-isomer, from **R** to **U'** there is also the same *H*-bond rearrangements that lead to the formation of the same species as in *Z*-isomer (iminium and boronate zwitterion), which can be confirmed by some distances registered from $\mathbf{TS_{T'\text{-}U'}}$ (depicted in Figure **24**): iminium ($d_{TST'\text{-}U'}$N–C = 1.29 Å) and boronate formation ($d_{TST'\text{-}U'}$B–O = 1.50 Å), with prior C-O bond cleavage ($d_{TST'\text{-}U'}$C–O = 2.26 Å). However, from this point on, there are significant changes in the two mechanisms that demonstrate a more thermodynamically favourable mechanism for the *E*-isomer.

In the fourth step of this path, from **U'** to **V'**, the second proton from the iminium ion is transferred to one of the hydroxyl groups coordinated with the boron atom, which is supported by the neighbour water molecule. Thus, in **V'** boron is coordinated to the aromatic substituent, two hydroxyl groups and the resulting water molecule. This protonation results in a clear weakening of the B-$\underline{O}H_2$ bond, as confirmed by the corresponding bond lengths (1.50 Å, in **U'**, and 1.64 Å, in **V'**).

After a second rearrangement of the *H*-bonds involving the water molecule, there is loss of a water molecule by uncoordination with the boron atom, and consequently iminoboronate formation **N''**. In the corresponding transition step, $\mathbf{TS_{W'\text{-}N''}}$, cleavage of the B–$OH_2$ bond is finished, as shown by the corresponding distance (2.72 Å).

From **N''** to **X** there is a final rearrangement of the *H*-bonds, involving two different water molecules, one being the solvent molecule present in the molecule since the beginning of the mechanism and the other corresponds to the reaction's side product obtained in the previous step (from **W'** to **N''**).

Similarly to the previous mechanisms calculated for the *E*-isomer, in the last step of this mechanism, from **X** to **O**, there is an internal geometry adjustment that corresponds mainly to a rotation around the NC-C(phenyl) bond. This leads to a close proximity between the imine's *N*-atom and the boron, thus establishing a N-B bond (d_ON-B= 1.71 Å) greatly enhancing the stability of product **O**. In the same line with the previous hypothetic mechanisms 1 and 2, this last step is strongly exo-energetic (ΔE = –7.4 kcal/mol) demonstrating an increase of stability due to N-B bond formation, which also reflected in an overall energy balance of –10.0 kcal/mol, which is indicative of a thermodynamically favourable reaction.

Finally, the energy activation calculated for this reaction (12.4 kcal/mol) is in agreement with the experimental conditions employed. This energy corresponds to the highest barrier along the path (from **T'** to $\mathbf{TS_{W'\text{-}N''}}$) which is related with one of the most difficult steps along the entire path – the second proton transfer between N–H to O–H. Despite the assistance of the solvent molecule in both proton transfer, the corresponding transition states $\mathbf{TS_{Q\text{-}R}}$ and $\mathbf{TS_{W'\text{-}N''}}$ are both 5.9 kcal/mol above the reactants **P**.

Once again, it is important to highlight the crucial role of boronic acid along the mechanism. As before, the boronic acid group started by establishing a B-O bond with the carbonyl's oxygen, promoting the nucleophilic attack from the amine's *N*-atom. Further along, respecting the true nature of this mild Lewis acid, it coordinates a hydroxyl group that is retained throughout the mechanism (from **U'** to **W'**). In the end, it has promoted two protonation steps, assisting the formation and loss of the water molecule (the leaving group in this reaction).

By comparison with the aboronated species or the mechanism between 1-butylamine and 4-ABBA, it is clear that this boron assistance along the reaction justify a significantly smaller energy barrier for the formation of the iminoboronate **O** (**89**), from 35-36 kcal/mol to 12.4 kcal/mol as aforementioned. Moreover, both mentioned mechanisms without N-B interaction led to slightly endo-energetic reactions (ΔE = 3–5 kcal/mol) versus the one where N-B interaction exists. In such case a strongly exo-energetic reaction (ΔE = –7.4 kcal/mol) was determined suggesting a reversible character for the first two species and an increase of stability. Such statements clearly justify the iminoboronate smooth formation in aqueous media.

Finally, the observed iminoboronate stability towards hydrolysis or in other words, the diminished reversibility of the corresponding reaction when compared with simple imine formation is a direct consequence of the N-B bond established between the boron atom and the imine *N*-atom, as shown by

a 7.4 kcal/mol energy gain in the final product (**O**) compared with intermediate **X** where the N-B bond is absent. In the optimized structure of **O** (**89**) the presence of the N-B bond is represented by a distance of 1.70 Å and it is confirmed by a Wiberg index of 0.48, indicative of a covalent bond (Figure **25**). Such observations support the stability of the product as much as the role of the boronic acid along this mechanism.

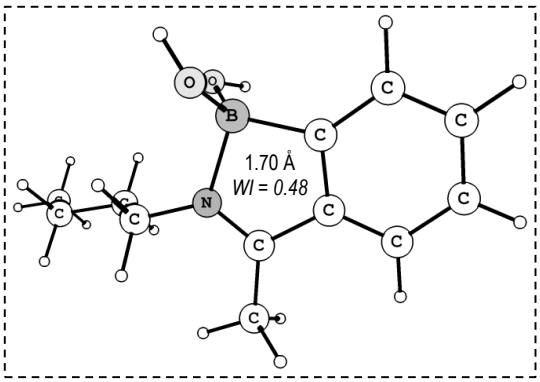


Figure **25** – Optimized geometry (PBE0/6-31G**) of the iminoboronate **O** (**89**). The N-B bond distance (Å) and the corresponding Wiberg index (WI, italics) are indicated.

II.4. Reversibility

Since the theoretical elucidation of the mechanism and stability of the final product was established by means of DFT calculations and it was in agreement with the experimental results, demonstrating an expected bioconjugation based on the formation of hydrolysis-stable iminoboronates, the following step relied on studying the possibility of controlled reversibility.

As aforementioned, it was envisioned that N-B dative bond could be disrupted by the influence of an external molecule, offering a mechanism to promote the imine hydrolysis and thus, revert the protein's modification. With that purpose in mind, several endogenous molecules (glucose, fructose, lactose, sucrose, ephedrine, thymine, adenine, cytosine, dopamine and glutathione) were added to iminoboronate **89** and a kinetic ^{1}H-NMR evaluation was performed.

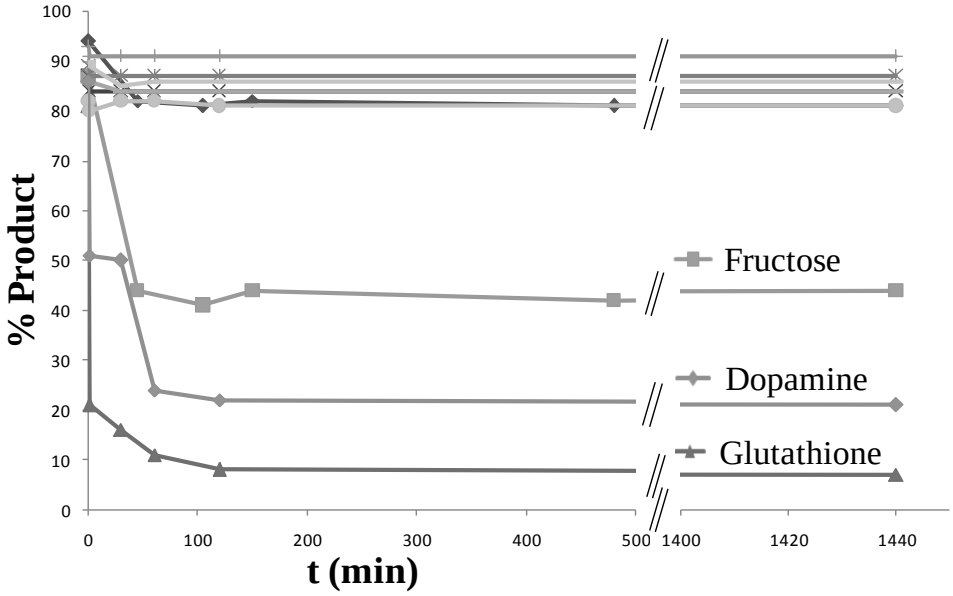


Figure **26** - Reaction of iminoboronate **89** with 1 equivalent of endogenous molecules (EM): glucose, fructose, lactose, sucrose, ephedrine, thymine, adenine, cytosine, dopamine and glutathione.

As shown in Figure **26**, most of the molecules tested had an almost negligible effect on the percentage of the preformed imine. Nevertheless, fructose and dopamine considerably reduced the concentration of iminoboronate **89** in 56% and 79% respectively, probably due to the formation of boronate esters which increased the electrophilicity/Lewis acidity of the boronate and thus amplified solvent insertion. Furthermore, glutathione decomposed the iminoboronate **89** in up to 7% in less than 2 h. This constitutes a very promising result when envisioning the design of delivery systems into cells because in the cell's cytoplasm glutathione exists in an increased concentration (milimolar range) and this fact may be targeted to promote the conjugate dissociation only when internalized.[39]

Taking in consideration the aforementioned results, the hydrolysis potential on biomolecules was evaluated using the well-established 2-FBBA-Lyz conjugates in an ammonium acetate buffer solution (20 mM) at pH 7.0. After mass spectrometry evaluation of these constructs, 1 equivalent of dopamine (**102**), fructose (**101**) and glutathione (**100**) were added separately to 2-FBBA-Lyz conjugates and the results obtained with ESI-FTICR-MS were recorded immediately (Figure **27**). Similarly to what occurred when using the model iminoboronate **89**, the addition of dopamine, fructose and glutathione rapidly induced the hydrolysis of the constructs. These results clearly support a controlled reversible modification of primary amines, which can be used as a new tool for protein modification.

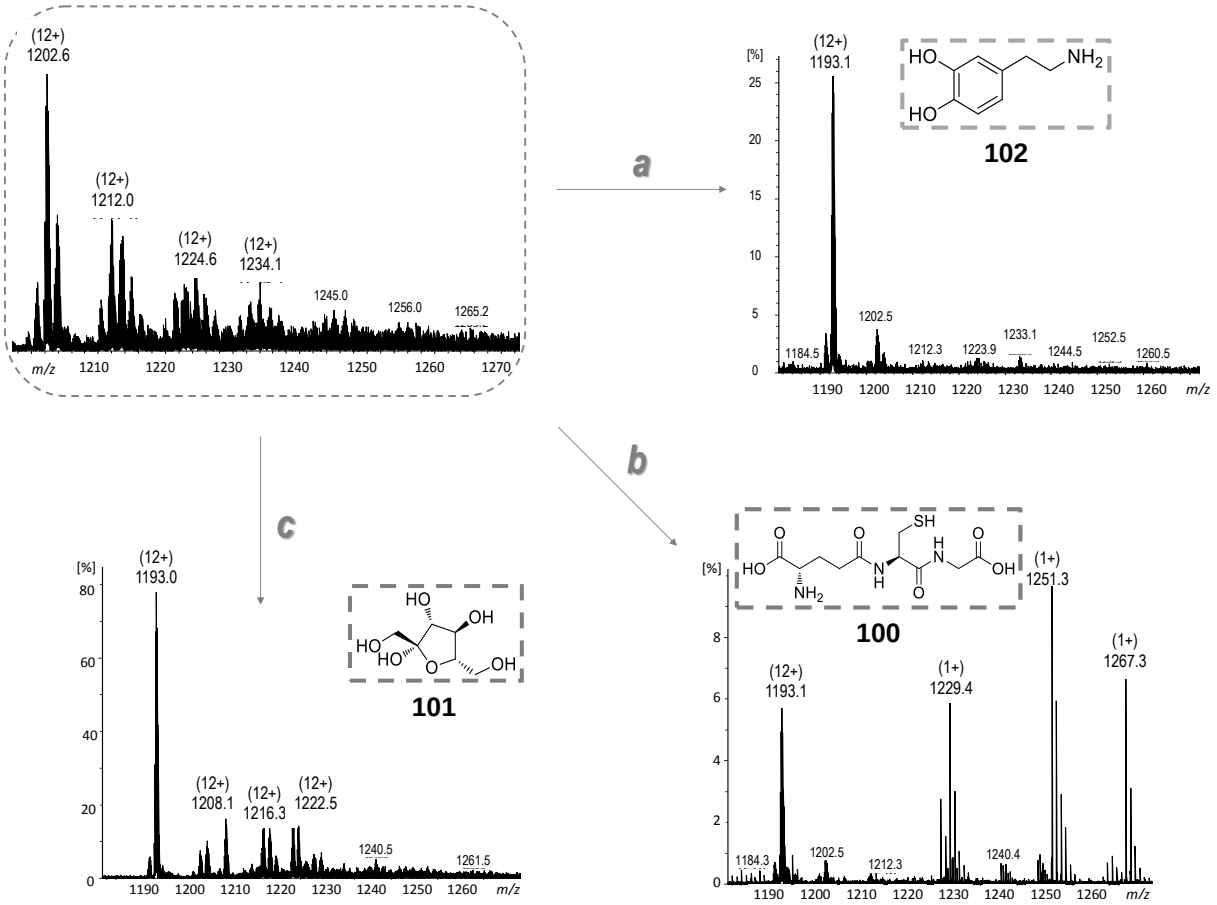


Figure **27** - Reaction of lysozyme constructs with equimolar amount of endogenous molecules (EM): dopamine, fructose and glutathione. A zoom of the ESI-MS spectra of the lysozyme constructs is displayed. The ESI-FTICR-MS spectra *a, b* and *c* were recorded immediately after the addition of dopamine, fructose and glutathione, respectively.

II.5. Conclusion

In the end, an innovative strategy to modify a protein's lysine ε-amino and *N*-terminal groups based on the generation of stable imines in aqueous media with 2-carbonylbenzeneboronic acids was presented. Several experimental data, as well as theoretical elucidation of the reaction's mechanism and on the hydrolytic stability of iminoboronates was demonstrated, which led to the fast and efficient modification of different biomolecules. Moreover, these iminoboronates allow a controlled reversibility upon the presence of certain endogenous molecules, such as glutathione, dopamine and fructose.

Chapter III

Modification of the novel modifying agent

ABSTRACT

Boronic acids and derivatives are sensitive compounds that entail certain handling requirements and, thus, though different methods are reported, modifications on their structure or their ground synthesis are difficult processes that often require different protection and deprotection steps.

In this chapter, different attempts to obtain 2-carbonylbenzeneboronic acid derivatives will be presented leading to a strategy that started from the incorporation of a small linker, followed by a required triflation reaction to perform a Miyaura-borylation. Hydrolysis steps as deprotection procedures were also required to generate a modifiable boronated backbone that could be changed depending upon the intended application.

Chapter III.
Modification of the novel modifying agent

III.1. Introduction

III.1.1. Chemical Properties of Boronic Acids

Since the boronic acid moiety plays a major role in iminoboronate conjugation system and it has specific chemical properties that could drive the synthesis towards certain paths over others, boronic acid properties and chemical reactivity will be explained in detail.

This chemical group presents a weak electron-withdrawing inductive effect due to the electronegative differences between boron and carbon (B = 2.05 and C = 2.55) and also a electron-withdrawing resonance effect due to the electronic deficiency of boron. Moreover, either aldehydes or ketones are electron-withdrawing groups and moderately deactivating groups, which means that these groups present an synergic effect with boronic acids, deactivating the aromatic ring.[233]

In terms of handling, most compounds bearing boronic acids can be handled in air, at room temperature and often these compounds present long shelf-stability. However, since these reagents are prone to atmospheric oxidation and autoxidation, they should be stored under an inert atmosphere. When dehydrated, either with a water trapping agent or through co-evaporation or high vacuum, boronic acids tend to form cyclic and linear oligomeric anhydrides such as trimeric boroxines (Figure **28**). Usually this is not an issue when used as synthetic intermediates (as they commonly are), however, for the desired synthetic routes of this project this property assumes a crucial relevance. [233]

Furthermore, upon exposure to air, dry species may decompose rapidly, and boronic anhydrides are considered to be initiators of the autoxidation process. For this reason, for long-term preservation it is advised to store these species under a slightly moist environment, as it happens for commercial samples. As an alternative, boronic ester are common surrogates of boronic acids that avoid anhydride generation.[233]

Therefore, relevant for the synthetic routes to be described one should highlight that 2-carbonylbenzeneboronic acids might present a slightly deactivated ring and should be handled under

inert and slightly moist atmosphere to avoid oxidation processes. Furthermore, dried samples can not be left under ambient air with no special precautions, since they readily form the corresponding alcohols through a process of atmospheric oxidation (Figure **28**).

III.1.2. Chemical Stability of Boronic Acids

The already mentioned oxidation processes only take into account boronic acids' inherent properties and not their chemical stability towards external agents and conditions. Three processes that these species suffer from should be highlighted: ligand exchange and disproportionation, chemical oxidation and protolytic deboronation.[233]

Regarding the first two processes that can occur while handling boronic acids, it is noteworthy that the substitution of the carbon-containing group of these species with other substituents is a slow process and thermodynamically unfavoured, which makes it very rare to find the disproportionation products (trialkylborane, borinic acid or boric acid) that are represented in Figure **28**. Moreover, concerning the second process involving boronic acid chemical oxidation, the oxidative cleavage of the C-B bond of boronic acid derivatives with water (under a wide pH range) or oxygen is a kinetically slow process. Nevertheless, under specific conditions and potent oxidizing agents, boronic acids and derivatives are readily oxidized (Figure **28**).[233]

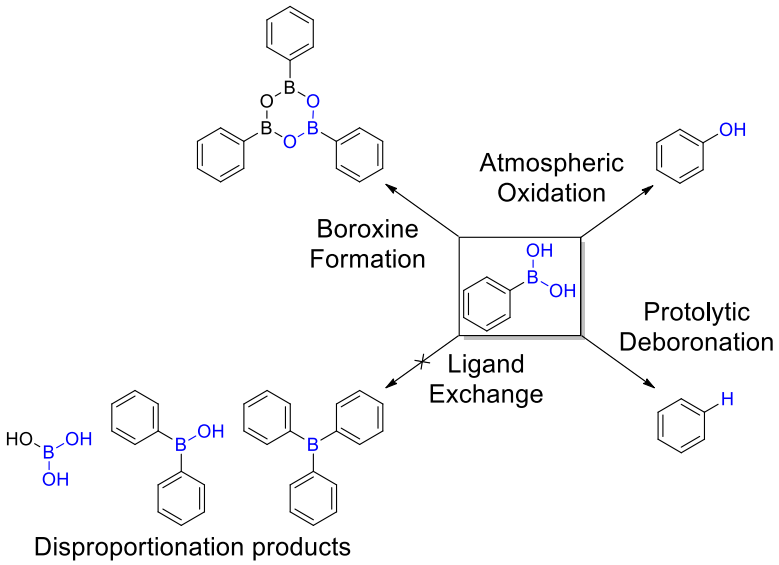


Figure **28** – Boronic Acids' Stability and degradation patterns

Finally, regarding the protolytic deboronation of boronic acids in aqueous media, the C-B bond is known for being highly protolytic resistant under neutral aqueous solutions, even at high temperatures. However, under highly acidic or basic aqueous conditions arylboronic acids can be promptly deboronated. Moreover, *ortho*-substituted and especially electron-poor arylboronic acids are recognized for their tendency to protodeboronate under basic aqueous conditions. The motif under study, 2-carbonylbenzeneboronic acids, is precisely a good example of such structure. In the case of electron-rich arylboronic acids, they tend to deboronate faster in strongly acidic aqueous media.[233]

Generally, arylboronic acids or esters tend to be very resistant to non-aqueous acids, but can be protodeboronated by means of metal-promoted C-B bond cleavage. In this regard, an order of effectiveness among metals that can perform aqueous protodeboronation was established: Cu (II) > Pb (II) > Ag (I) > Cd (II) > Zn (II) > Co (II) > Mg (II) > Ni (II), though other metals may promote the same transmetallation-protodeboronation, like Hg (II), Rh (II) or even Pd (0). [233]

Thus, to summarize, *ortho*-substituted and electron-poor arylboronic acids (such as 2-carbonylbenzeneboronic acids) have a competitive protolytic deboronation which is exacerbated by the basic conditions and the use of transition metals.

III.1.3. Synthesis of Boronic Acids

With the objective of delineating a synthetic strategy to derivatize 2-ABBA/2-FBBA, a useful alternative to produce different 2-carbonylbenzeneboronic acid species could arise from modifications of aboronated species with the incorporation of the boronic acid in a final stage of the synthesis. For that reason, borylation methods will be discussed below.

Many different methods have been reported to introduce the boronic acid functionality, though the most used ones involve the electrophilic trapping of arylmetal intermediates with borates from aryl halides or from directed *ortho*-metallation; the transmetallation of arylsilanes and arylstannanes; the transition metal-catalyzed coupling between aryl halides/triflates and diboronyl reagents or the direct boronylation by transition metal-catalyzed aromatic C-H functionalization.[233]

Since the older borylation methods were based on hard organometallic species or generated from hard boron halides which required rigorously anhydrous conditions to be performed, the transition metal-catalyzed coupling between aryl halides/triflates and diboronyl reagents seemed to be the most promising methodology to obtain the sought compounds. Thus, since diboronyl esters, such as bis(pinacolato)diboron (B_2pin_2), have been described as agents to perform a smooth palladium catalysed

cross-coulping reaction with aryl groups bearing good leaving agents, this protocol seemed to be a valid choice to pursue the strategy that started from aboronated species. Moreover, the scope of halo or triflatearene substrates in these reactions is very broad, even having described methods for *ortho*-carbonylaryl derivatives, which would be unsuitable for any procedure using organolithium or organomagnesium intermediates without resorting to protection steps. Standard conditions for this coupling reaction involve, apart from the already mentioned diboronyl esters, $PdCl_2$(dppf) as catalyst, potassium acetate as base and a polar aprotic solvent as the reaction medium.[233]

III.2. Goals

Hence, in this chapter, different strategies to obtain 2-carbonylbenzeneboronic acid derivatives will be presented, starting from modifications to 2-FBBA core and 2-ABBA core, in an attempt of having hydroxyl or amine derivates without performing any borylation methods and using the compounds already available in our inventory.

Since this methodology proved to be inefficient, different strategies based on borylation methods were developed, though, in all cases, the borylation step proved to be the most crucial synthetic step of the strategies developed. In this chapter, all limitations that arose while developing borylation methods will be commented as well as all the solutions to overcome those problems.

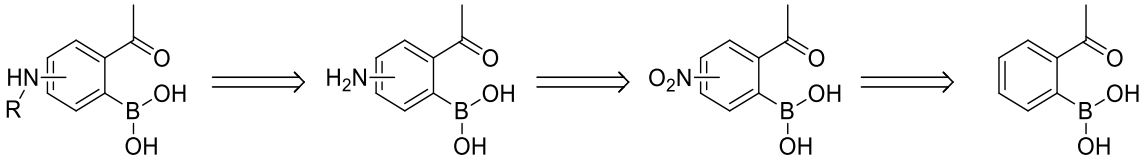
In the end, it will be presented a backbone featuring an acid function that could be early modified through amidations or esterifications, depending upon the purpose and the starting molecule. These last reactions will only be presented in **Chapter IV.**

III.3. Strategies to achieve a modifiable backbone

III.3.1. Modification of 2-carbonylbenzeneboronic acid

Modification of 2-carbonylbenzeneboronic acids was attempted as a strategy to avoid borylation methods and to take advantage of commercially available reagents that bear this motif. First, an attempt to modify the 2-ABBA core was experimented, through an aromatic nitration of this unprotected structure and then, a methylation of a boronate ester derivative of 2-FBBA was also endeavoured to take advantage of all the *ortho*-formylbenzeneboronic acids available in the laboratory.

Nitration of some boronic acids has been reported and, based on one of those procedure, nitration of 2-ABBA was attempted first. The retrosynthetic route outlined started from this reaction, which was the most crucial step of this strategy, and then move on (with our without protecting the carbonyl group) into a well-known reduction of the nitro group to afford amines. And, in the end, the last step would be based on the functionalization of this amine-derivative of 2-ABBA using halogen derivatives to perform S_N2 displacement of the halogens to afford secondary amines, under basic conditions (Scheme **35**).



103 **104** **105** **88**

Scheme **35** – Retrosynthesis of amino-derivatives of 2-ABBA

The first attempt on performing a nitration of 2-ABBA was based on a procedure that started from *meta*-amidederivatives of benzeneboronic acid, which seemed as a good starting point to nitrate 2-ABBA. As a first result, though a species with only three aromatics protons was isolated (as expected from compound **105**), it did not present any alkylic proton signals (Scheme **36**).

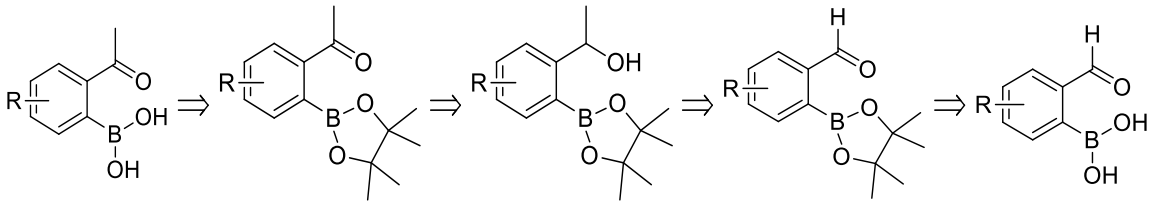
88 **105**

Scheme **36** – Nitration attempt of 2-acetylbenzeneboronic acid (**88**)

In another attempt at the same reaction, complete conversion of 2-ABBA was again detected by TLC, though, in this case, a different species was obtained with three other aromatic proton signals. Nevertheless, the major isolated products were unreacted 2-ABBA or a compound with four protons that could be the result of a protolytic deboronation.

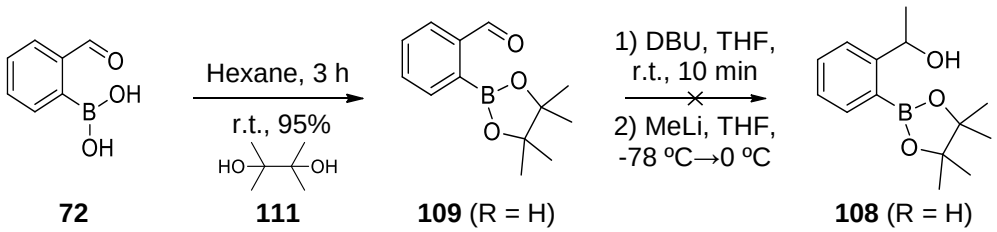
In the end, this strategy was dismissed due to results on infrared spectroscopy and mass spectrometry that, in any case, demonstrated none of the expected bands or masses for compound **105**.

In a second attempt to obtain 2-ABBA derivatives, a strategy was outlined to take advantage of commercially available 2-FBBA. First, since organometallic agents were to be used in order to perform the methylation of the formyl group, a first protection of 2-FBBA was required to avoid any interference. Next, the most critical step of this strategy would be performed using methyllithium in THF at low temperatures, based on a reported procedure for aldehydes conjugated with alkenylboronic acids. Then, an oxidation would be performed using mild oxidizing agents selective towards secondary alcohols and lastly a simple deprotection step would be required to generate the envisioned 2-ABBA derivatives (Scheme **37**).



Scheme **37** – Retrosynthesis of 2-ABBA derivatives from commercially available 2-FBBA

Therefore, to follow the described retrosynthesis, the first protection step was performed with excellent yield. Then, different attempts on the methylation of the pinacolyl ester of 2-FBBA were attempted. However, no conversion was observed (Scheme **38**), and thus, this strategy was rapidly discarded as a feasible possibility.



Scheme **38** – Methylation attempt of 2-formylbenzeneboronic acid (**72**)

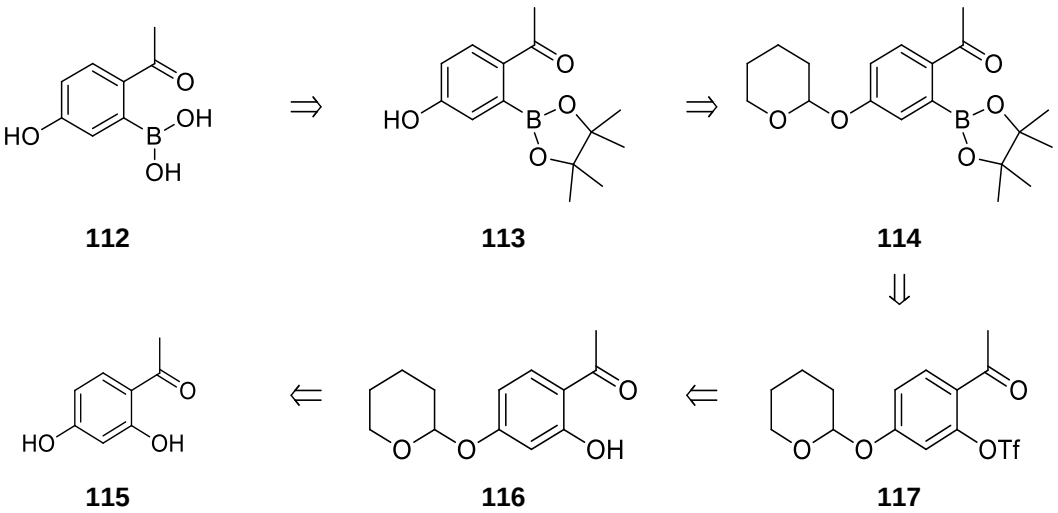
Modifications of 2-carbonylbenzeneboronic acids proved to be very difficult to evaluate, since all available analytical techniques offered uncertain results impairing their interpretation. Boronic acids' presence was a constant interrogation during these modification steps due to the possibility of protolytic deboronation, as explained in the introduction. Therefore, methods that introduce boronic acids further along the synthesis seemed to be a more attractive strategy.

III.3.2. Construction of 2-carbonylbenzeneboronic acid

As mentioned in the previous topic, new synthetic methodologies that incorporated the boronic acid moiety in later steps of the synthetic path had to be developed. This strategy appeared to be more reasonable taking into account the difficulty found in working with boronic acid derivatives. Thus, the final goal was to find a core molecule that could be modified depending upon the intended biological application. To this end, three different strategies were studied aiming at the synthesis of these compounds.

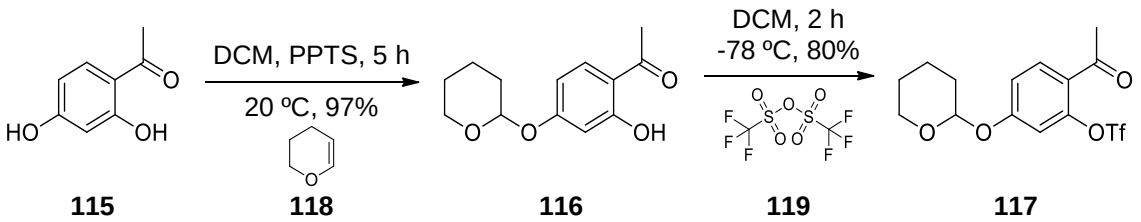
III.3.2.1. Strategy 1

Based on the aforementioned the first retrosynthetic pathway considered for our target compound is depicted in Scheme **39**, which started from 2',4'-dihydroxyacetophenone (**115**) to ultimately generate 5-hydroxy-2-ABBA (**112**). Compound **112** can then be further modified *via* phenol alkylation to yield different derivatives of 2-ABBA. Five consecutive steps would be required to obtain such molecules, starting from the selective protection of the *para*-hydroxyl group of **115**, then, a triflation of the remaining hydroxyl group would yield a substrate (**117**) for the Miyaura-borylation. This was envisioned to be the crucial step of this synthetic pathway, since only deprotection procedures remained to be performed and they are believed to be simple steps (Scheme **39**).



Scheme **39** – Retrosynthesis of 5-hydroxyl-2-ABBA

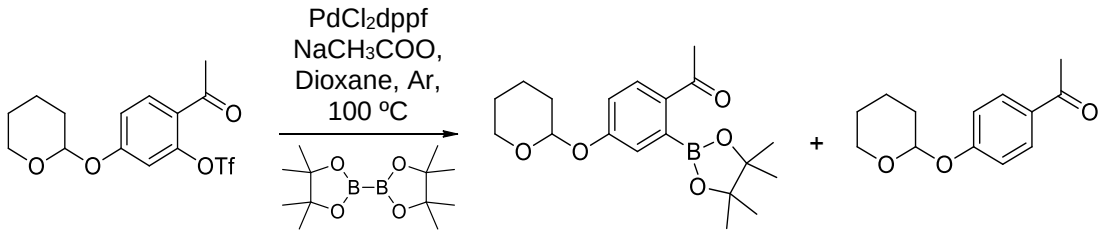
As expected, the first two steps were quite straightforward and reproducible (Scheme **40**). In different attempts made, the protection with 3,4-dihydro-*2H*-pyran (**118**) gave excellent yields (from 93% to 97%) and the triflation with triflic anhydride (**119**) afforded compound **117** in good yields (from 75% to 80%). Then, the borylation reaction was studied.



115 **118** **116** **119** **117**

Scheme **40** – PTS protection and subsequent triflation of 2',4'-dihydroxyacetophenone

Protolytic deboronation was a major issue during this borylation process (Scheme **41**), supposedly due to an interaction between the nearby oxygen from the acetyl group and the boron atom, making C-B bond more labile. Thus, after several unsuccessful attempts to perform the borylation with the standard conditions, product **114** was finally isolated but only in 6% yield. Then, we proceeded with the optimization of the reaction conditions. With this goal in mind, different palladium sources, ligands, bases and solvents were tested as shown in Table **11**. In all cases, all reagents were dried and solvents distilled in order to have pure starting materials. Considering the results obtained with this screening, freshly distilled dioxane, sodium acetate, $PdCl_2$(dppf) and bis(pinacolato)diboron (**120**) proved to be the most suitable system to perform this reaction. Despite this, even under the optimized reaction conditions, compound **114** was obtained in only 40% isolated yield, alongside with 43% isolated yield of **121**.



117 **120** **114** **121**

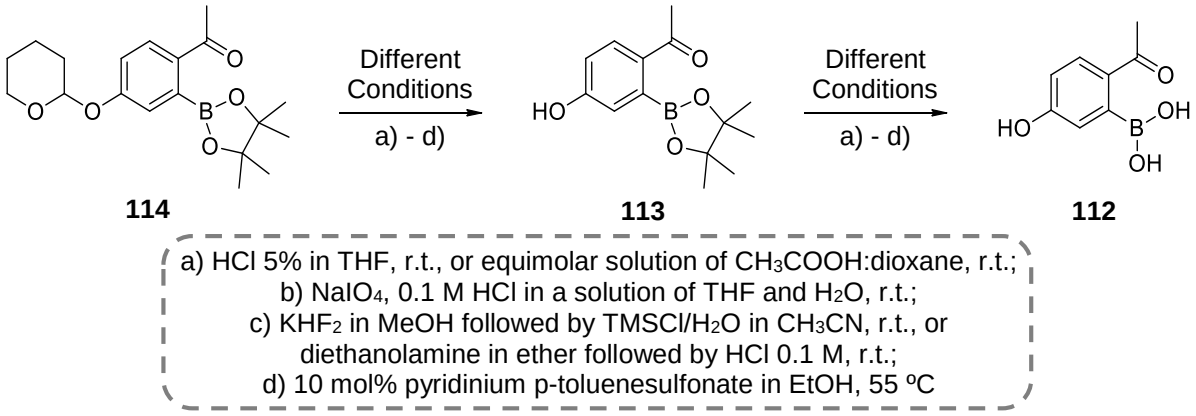
Scheme **41** – Miyaura-borylation of compound **117**

Table **11** – Optimization attempts on Miyaura borylation. The best results are in bold.

Solvent	Palladium	Ligand	Base
Toluene	Pd (II) chloride	Triphenylphosphine	KCH_3COO
Dioxane	Pd (II) acetate	(R)-BINAP	Na^tOBu
MDE	Pd (II) acetylacetonate	(R)-DIOP	K^tOBu (sublimed or not)
	Pd (II) trifluoroacetate	Tributylphosphine	TEA
	$PdCl_2$(dppf)	2,2'-bipyridin	K_2CO_3
	Tris(dibenzylideneacetone)dipalladium (0)		**$NaCH_3COO$**
			NH_4CH_3COO

Despite many attempts, this proportion of 1:1 between the borylation product (**114**) and the protolytic deboronation product (**121**) was never overcome. Despite those unsettling results, the remaining retrosynthetic path was still attempted.

Based on reported procedures for the deprotection of THP or pinacolyl esters, different chemical deprotection strategies were attempted. These included the use of strong acids, oxidizing agents and specific protocols to selectively deprotect the pinacolyl ester or the THP independently (Scheme **42**).



Scheme **42** – Deprotection steps of compound **114**

The use of strong acids, generally, deprotected THP ether and originated **121** in considerable yields. The methodology using oxidizing agents such as $NaIO_4$ gave a mixture of polar products that proved to be impossible to separate and the compound **121** as the major product.

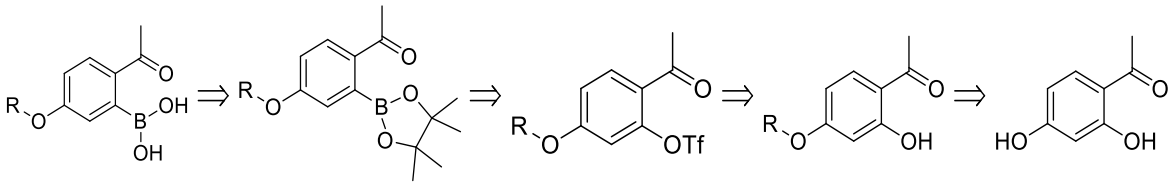
The first methodology to specifically deprotect pinacolyl boronate ester with the 2 step procedure was very effective, though the THP ether remained unaffected and a considerable amount of the protolytic deboronation product (**121**) was fomed. The second methodology with diethanolamine afforded a large number of products that were not isolated.

The final methodology to deprotect THP ether selectively was very effectively achieved using an acidic catalysis in ethanol and afforded compound **113** in good isolated yields (80-85%). Unfortunately, subsequent pinacolyl boronate ester deprotection always afforded the protolytic deboronation compound (**121**) as a major product.

III.3.2.2. Strategy 2

Taking into consideration the difficulties encountered in the above mentioned strategy, an alternative protocol was designated to prepare our target compounds.

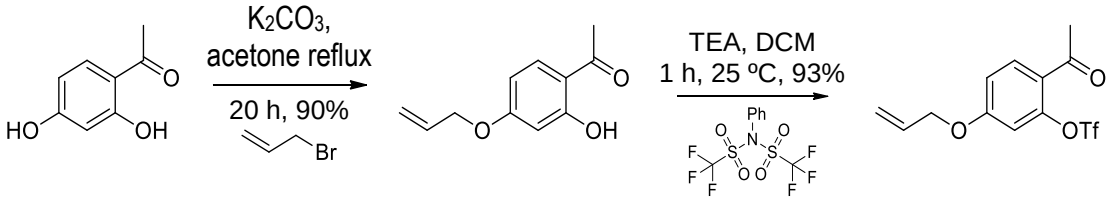
The envisioned retrosynthesis is shown in Scheme **43** and starts from alkylation of 2',4'-dihydroxyacetophenone to install an alkenyl and alkynyl function, then it follows the same steps described previously. Installing an alkenyl and a terminal alkynyl function permits different options to perform the functionalization of the scaffolds based on very efficient click reactions such as TEC or CuAAC, or other selective reactions, like Grubbs-metathesis. First, all results regarding the alkenyl moiety will be presented, followed by the results with alkynyl moiety.



122 **123** **124** **125** **115**

Scheme **43** – Retrosynthesis of clickable-2-ABBA, R bears an alkenyl or alkynyl group

When using 3-bromo-1-propene (**126**) as the alkylating agent, the first two steps of the synthesis were quite simple and reproducible (Scheme **44**). In different attempts made, the reaction with the allyl bromide afforded very good yields of the alkylated product **125a** (from 80 to 90%) and the triflation reaction with *N*-phenylbis(trifluoromethanesulfonimide) yielded the expected compound **124a** in excellent yields (from 90 to 93%).

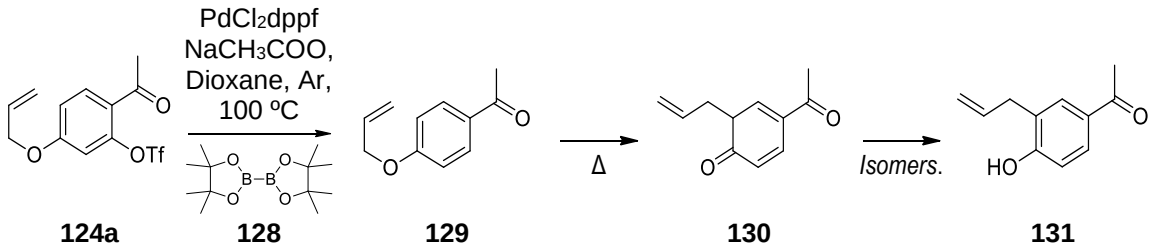


115 **126** **125a** **127** **124a**

Scheme **44** – Alkylation with 3-bromo-1-propene and subsequent triflation of 2',4'-dihydroxyacetophenone

Once again, the Miyaura-borylation step proved to be considerably more difficult and despite several attempts the boronated species was never isolated. Typically this reaction afforded three major products: protolytic deboronation compound (**129**), a dealkylated triflate and a hypothetic Claisen rearrangement

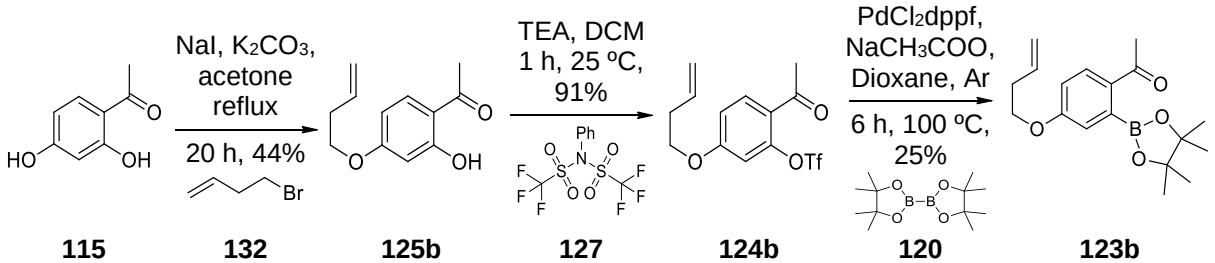
product (**131**, Scheme **45**). From the crude ^{1}H-NMR after 4 h of reaction it was possible to confirm the protolytic deboronation product was the major product.



Scheme **45** – Miyaura-borylation of compound **124a** and hypothetic Claisen rearrangement

Then, the same reaction was attempted but using 4-bromo-1-butene instead of 3-bromo-1-propene, which would generate a thermodynamically unfavourable seven-member ring intermediary that would not proceed to the Claisen rearrangement. Furthermore, this length increase of the alkylated chain would also avoid allyl ether cleavage promoted by transition-metals, like palladium.

Under the same conditions used in the previous example, the optimized alkylation step with 4-bromo-1-butene proved to be much less efficient and reproducible affording the product **125b** in low to moderate yields (from 20 to 44%). The triflation step with *N*-phenylbis(trifluoromethanesulfonimide) went smoothly yielding the product **124b** in excellent yields (from 88 to 91%). Very gratifyingly, the Miyaura-borylation was performed successfully yielding the expected product **123b** in 25% isolated yield (Scheme **46**). No dealkylated product was ever detected and the protolytic deboronation was the only secondary product identified in the reaction mixture. A kinetic ^{1}H-NMR study over 5 h of reaction proved that the protolytic deboronation product was formed at the same time as the boronated species (Figure **29**). Thus, the improvement of this reaction proved to be very difficult, affording the product in only 20-30% yield.



Scheme **46** – Alkylation with 4-bromo-1-butene and subsequent triflation of 2',4'-dihydroxyacetophenone

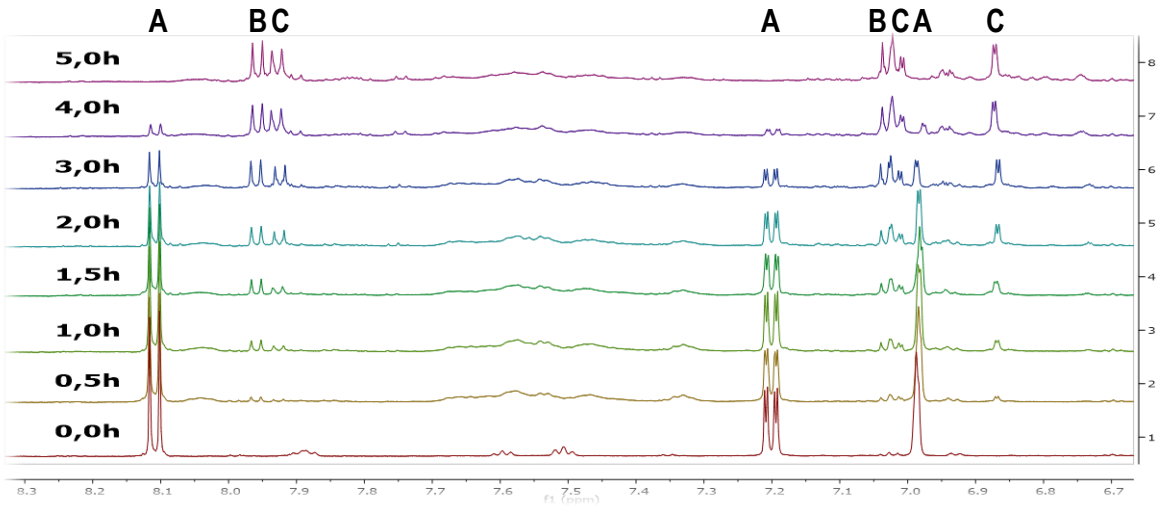
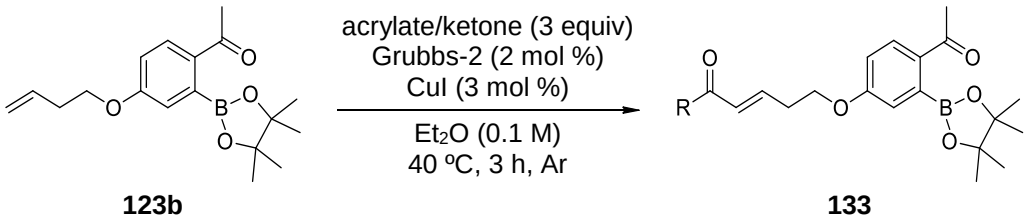


Figure **29** – Kinetic ^{1}H-NMR study of Miyaura-borylation from the alkene, **A** – Triflate compound **124b**; **B** – Protolytic deboronation compound (alkylated 4-hydroxyacetophenone); **C** – Boronated compound **123b**

Hence, modifications of the borylated alkene were attempted. Instead of using thiol-ene click chemistry that produces reactive thiyl radicals and may reverse the iminoboronate modification (if performed on the conjugated biomolecule), a known selective reaction for alkenes was attempted to further functionalize this product. Grubbs cross-metathesis was selected as a feasible reaction to be performed with this core structure. First, different simple models were experimented in order to outline the optimal conditions for this reaction and to test its feasibility while using boronate derivatives. Following a described procedure, depicted in Scheme **47**, methylvinylketone and vinyl acrylate were tested as models for the cross metathesis with compound **123b**, affording the expected products in good yields (65 and 68%, respectively). However, when more complex compounds were used, this reaction proved to be unreproducible.

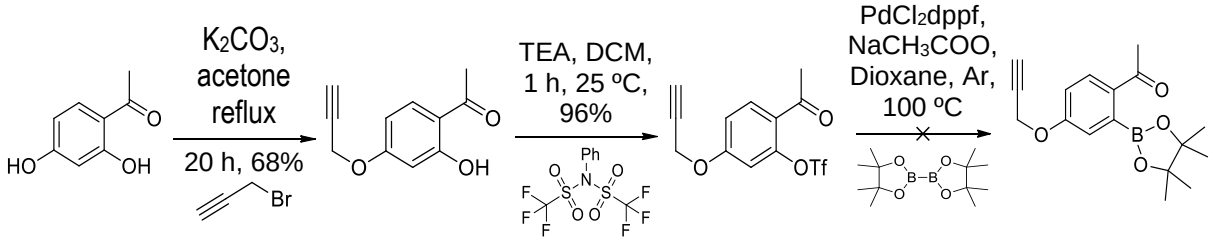


123b **133**

Scheme **47** – Grubbs cross-metathesis with compound **123b**

Considering the difficulties encountered in the previous synthesis, the installation of an alkyne function was attempted aiming to use this functionality in bioorthogonal reactions, such copper catalysed azide-

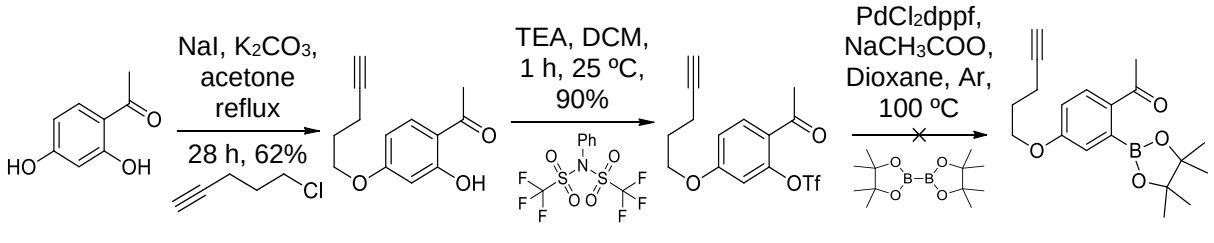
alkyne cycloaddition. Therefore, the alkylation of 2',4'-dihydroxyacetophenone was performed with propragyl bromide (**134**) under the same conditions, to afford 66-68% of product **125c**. Triflation of the monoalkylated species was the next step and, as before, afforded excellent yields of the expected compound **124c** (from 94 to 96%). Miyaura-borylation of this substrate was the problematic step of this methodology, never affording the expected boronated compound **123c** (Scheme **48**). The only isolatable product was the dealkylated triflate species. Consumption of the starting material was quickly detected by TLC, but no product was ever detected.



115 **134** **125c** **127** **124c** **120** **123c**

Scheme **48** – Alkylation, triflation and borylation starting from propragyl bromide and 2',4'-dihydroxyacetophenone

Consequently, since the dealkylation promoted by the palladium under such conditions could be the leading side reaction of this Miyaura-borylation, as before, an increase of length of the alkylic chain was used to test such hypothesis. Thus, another alkylation of 2',4'-dihydroxyacetophenone was performed, though this time with 5-chloro-1-pentyne (**135**) and sodium iodide which afforded the expected product **125d** in 62% yield. From this point, *N*-phenylbis(trifluoromethanesulfonimide) was again used to introduce the triflate moiety generating the product **124d** in very good yields (from 80 to 90%). Finally, the Miyaura-borylation was performed on this substrate, and despite several attempts, only the starting material was ever isolated. Based on the ^{1}H-NMR, the reaction after 2 h generated a very complex mixture, as shown by the spectra in Figure **30**. Thus, different strategies were designed to solve this situation.



115 **135** **125d** **127** **124d** **120** **123d**

Scheme **49** – Alkylation, triflation and borylation starting from 5-chloro-1-pentyne and 2',4'-dihydroxyacetophenone

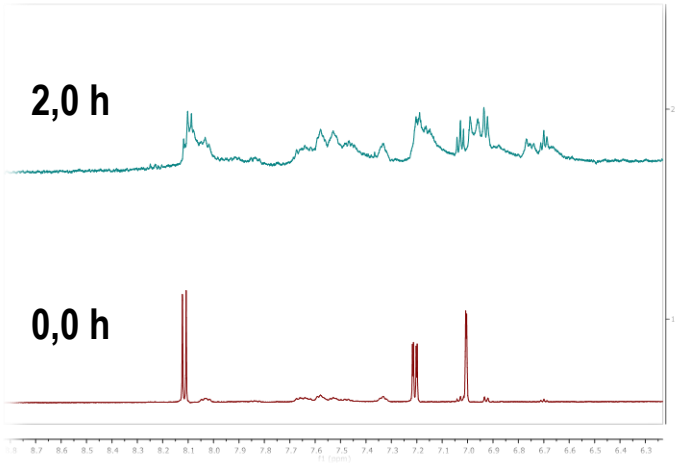
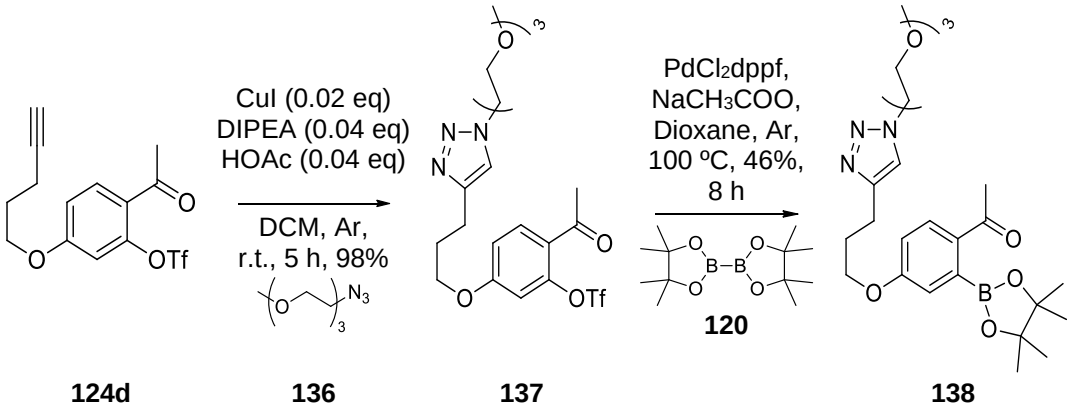


Figure **30** – Kinetic ^{1}H-NMR study of Miyaura-borylation from the alkyne **124d**

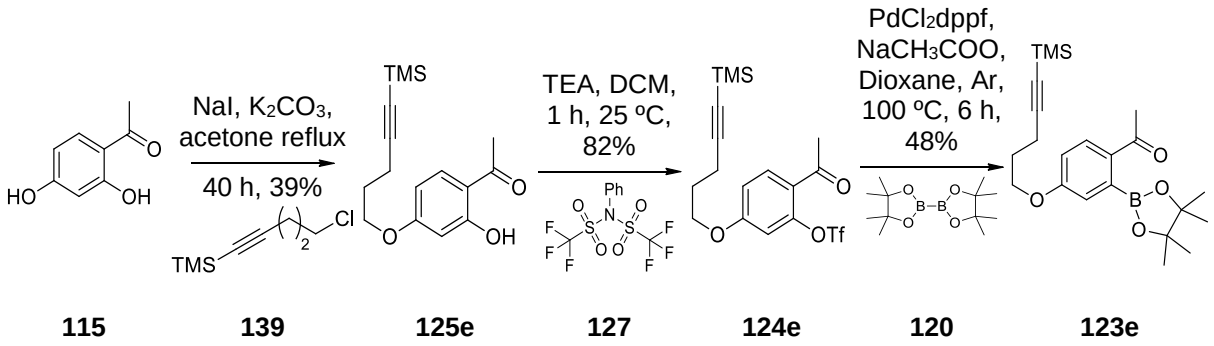
Based on these results we envisioned that the alkyne could be a possible source of complications *via* interactions with the catalyst. Therefore, two strategies were outlined to test this hypothesis: one, being the CuAAC performed in the triflate followed by the Miyaura-borylation and the second involving the use of a protected terminal alkyne.

As depicted in Scheme **50**, the first strategy was tested using tri(ethylene glycol)azide (**136**) which was reacted *via* CuAAC with compound **123d**. This reaction afforded the product **137** in 98% yield, which was submitted to the Miyaura-borylation to generate 46% isolated yield of a mixture of compound **138** and the resulting protolytic deboronation compound (in a 1:1 ratio). Thus, a proof of concept was established, highlighting that the unprotected terminal alkyne plays a crucial role in inactivating the triflate for the Miyaura-borylation. Nevertheless, this strategy was not further pursued since performing a different Miyaura-borylation in individual substrates designed for a specific application would not be a feasible methodology.



Scheme **50** – CuAAC and subsequent Miyaura-borylation starting from compound **123d**

Based on this, we tested the possibility to use protected alkyne functions, as depicted in Scheme **51**. Therefore, (5-chloro-1-pentynyl)trimethylsilane was used to alkylate 2',4'-dihydroxyacetophenone.This reaction afforded the product **125e** in moderate yields (40-48%). Then, a triflation of this compound generated the product **124e** in 82% yield. The borylation of this protected alkyne-triflate derivative was followed by ^{1}H-NMR over 6 h. This study showed that the desired product was formed, though the protolytic deboronation product was also generated (Figure **31**). In the course of the study, the borylated product **123e** was isolated in 48% yield, and this species was used to test the trimethylsilane deprotection. Unfortunately, this deprotection did not go as well as planned and apart from the unreacted starting material, the only isolated product without the trimethylsilane group did not have pinacol boronate ester.



Scheme **51** – Alkylation, triflation and borylation starting from (5-chloro-1-pentynyl)trimethylsilane and 2',4'-dihydroxyacetophenone

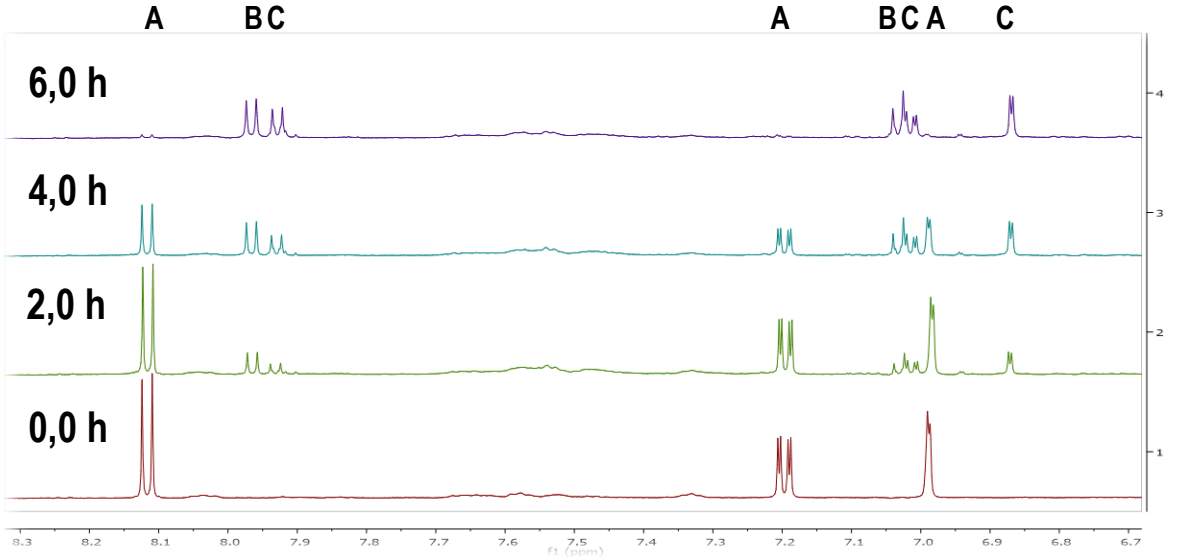
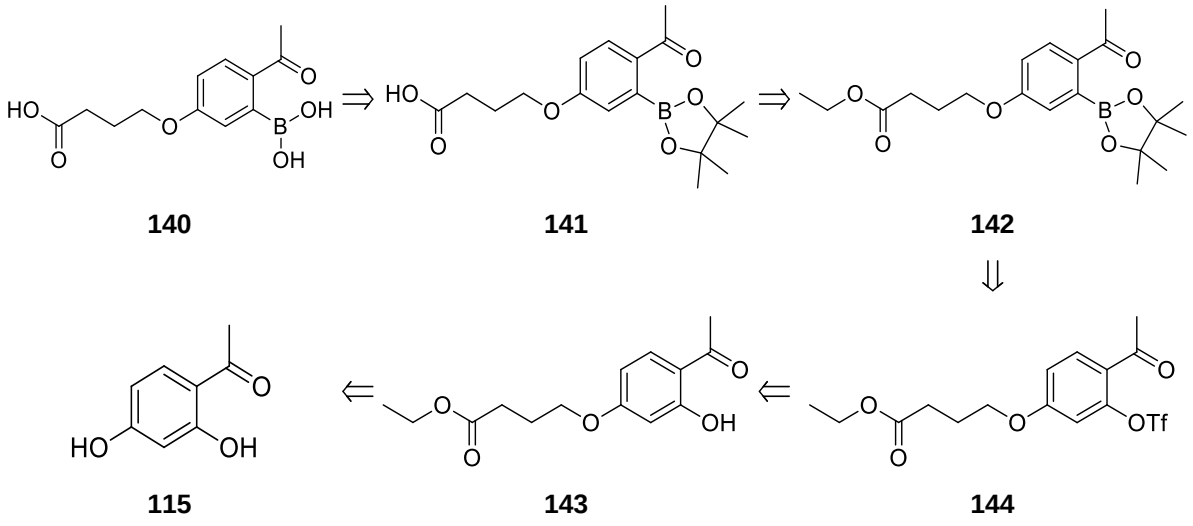


Figure **31** – Kinetic ^{1}H-NMR study of Miyaura-borylation from the protected alkyne**; A** – Triflate compound **124e**; **B** – Protolytic deboronation compound (alkylated 4-hydroxyacetophenone); **C** – Boronated compound **123e**

In the end, these studies aiming to prepare boronated compounds with biorthogonal handles that could be readily modified under native conditions did not afford the expected results. Furthermore, a likely insertion of palladium to generate organopalladium species with the alkene or the alkyne proved to be difficult to overcome.

III.3.2.3. Strategy 3

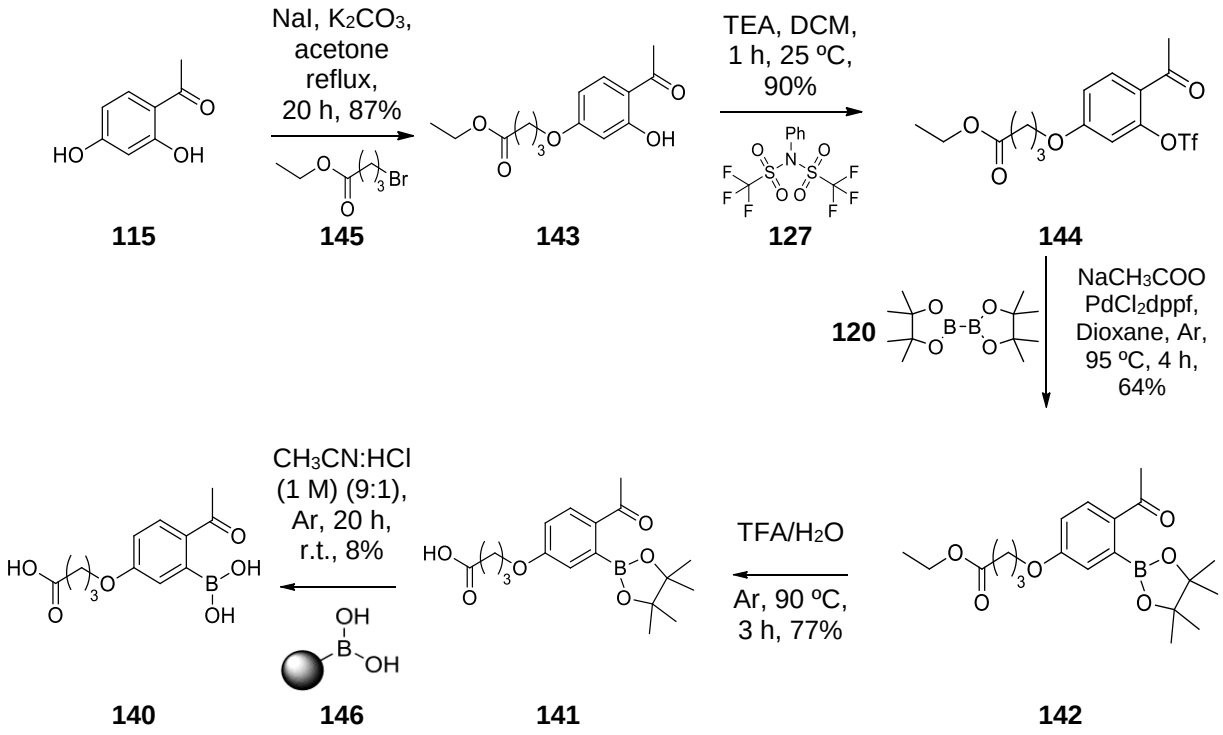
Since the previous delineated strategies proved to be impractical to prepare a core structure that may be further functionalized by means of simple chemical procedures, a different strategy was outlined as shown in Scheme **52**. In this case, the goal was to synthesize the boronated compound with a small linker featuring a carboxylic acid to undergo modifications with amines or alcohols. To achieve this, the procedure follows similar sequential steps as described before: alkylation, triflation and Miyaura-borylation. The borylation step is sensitive to the presence of an acid functionality, therefore this step will be performed with an ester instead of a carboxylic acid. For this reason, the last step will require a hydrolysis to generate the intended boronated carboxylic acid-derivative (Scheme **52**).



Scheme **52** – Retrosynthesis of 2-ABBA acid derivative

As depicted in Scheme **53**, 2',4'-dihydroxyacetophenone was selectively alkylated with ethyl 4-bromobutyrate (**145**) several times, under different conditions, with yields ranging from 50 to 87%. This wide variation was justified with the optimization of such step by decreasing the number of equivalents of sodium iodide from 2 to 1. This reagent was performing a competitive reaction that inhibited the desired alkylation of the hydroxylated ketone. After this, a triflation with *N*-phenylbis(trifluoromethanesulfonimide)

afforded **144** in very good yields (from 87 to 92%). Very gratifyingly, the Miyaura-borylation afforded the product **142** in the first attempt and after several attempts of optimization (mainly changing the time of the reaction from 4 to 30 h) in 64% of isolated product.



Scheme **53** – Total synthesis of compound **141**

Once the boronated compound was synthesized, the remaining steps to obtain a modifiable core structure were deprotection steps. The hydrolysis of the linear alkylic ester, after optimization, led to a reproducible reaction that afforded the product **141** in good yields (70-77%). Then, the second step also afforded the expected product through a transesterification procedure with a phenylboronic acid resin (**146**). Despite this, compound **140** was found to be difficult to isolate and exhibit a poor stability. Therefore the pinacol removal was only performed after the amidation or esterification of the carboxylic acid, immediately prior to its use in bioconjugation reactions.

III.4. Conclusion

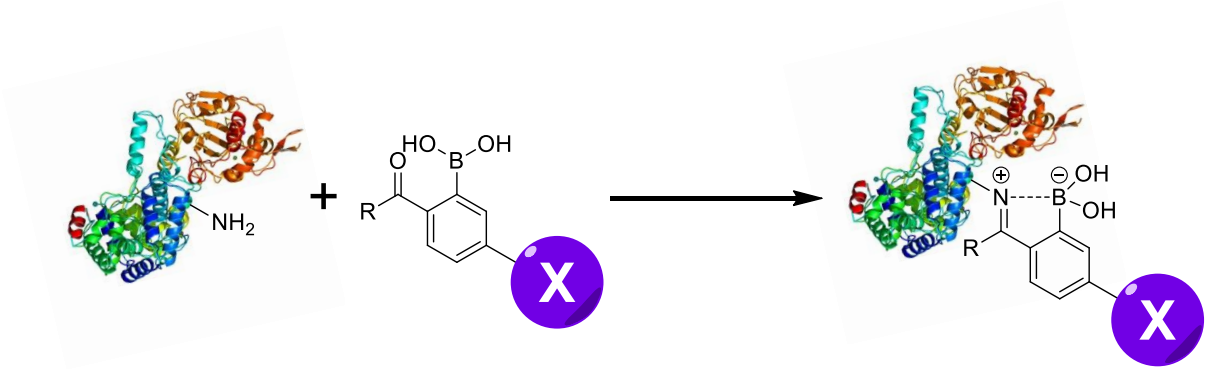
In this chapter, different attempts to obtain 2-carbonylbenzeneboronic acid derivatives were presented, leading to a strategy that started from the incorporation of a small linker, a required triflation reaction to perform a Miyaura-borylation and a final hydrolysis step to form a functionalizable carboxylic acid.

The Miyaura-borylation proved to be the most difficult step of all the strategies, often leading to the undesired protolytic deboronation product as a side product. Despite different attempts on optimization and all the purifications performed on the starting materials, different kinetic ^{1}H-NMR studies proved that 2-acetylbenzeneboronate pinacolyl ester generation and its deboronation occurred in parallel, probably due to a B-O interaction that weakens the newly formed C-B bond.

Nevertheless, in the end, a modifiable boronated backbone was obtained and used to further modification depending upon the intended application, as it will be discussed in **Chapter IV.**

Chapter IV

Applications



ABSTRACT

After the discovery and synthetic optimization to achieve the core molecule presented in **Chapter III.** different strategies to achieve different applications will be presented in this chapter.

Several compounds with different properties were synthesized and important biological studies were performed. Namely, an artificial glycosylation of a protein based on a click procedure (CuAAC), the installation of a fluorescent probe in a protein through another click procedure (SPAAC), the direct functionalization of a protein with two different fluorescent probes, several PEGylations of different biomolecules and, finally, the attachement of a cytotoxic drug to a vitamin.

From these bioconjugations, some led to important conclusions of the biological properties of the conjugates. Particularly, the fluorescent conjugations that led to the conclusion that iminoboronates are stable enough to go through cellular internalization, the sugar-dependent reversible PEGylation of insulin and the cytotoxic conjugate that accumulated preferentially in cancer cells vs healthy cells, in opposite to the free drug.

Currently, the study of intracellular reversibility of the conjugate is in progress through the development of an "ON-OFF" probe that is specific for this type of conjugation.

Chapter IV. Applications

IV.1. Introduction

As mentioned briefly in **Chapter I.** protein chemical modification enables a wide range of applications in different biological areas, mainly due to the large number of functions that these biomolecules can perform. These applications are most evident in the field of medicinal chemistry and led to the production of several new biomolecule-based drugs approved by different regulatory authorities. Nevertheless, before leading to such remarkable compounds, these applications started from mimicking nature's protein post-translational modifications, evolving then to the artificial enhancement of biomolecules.

IV.1.1. Post-Translational Modification mimetics

As mentioned in **Chapter I.** different post-translational modifications are crucial for the day-to-day operations of a living organism. These modifications have such a pivotal role in these organisms, that they can be used as criteria to distinguish some species over others, since the sites and regularity of PTMs on a given biomolecule can vary depending upon the living organism. Methylations, acetylations, phosphorylations, ubiquitinations and glycosylations were given as examples of PTMs, all of which can be artificially introduced by means of chemical modification of proteins.[6]

Artificial glycosylation is a particularly important protein modification that allowed the emergence of one of the most interesting applications of chemical modifications of proteins, which is the development of vaccine candidates. Artificial glycosylation of proteins can be used for several biological purposes, among which the development of synthetic glycoprotein vaccines candidates for different pathologies, since polysaccharide antigens can interact directly with B cells, inducing a boost of the immune response.[265] This strategy usually involves a carbohydrate antigen that is conjugated to an immunogenic carrier (*e.g.* BSA or keyhole limpet hemocyanin – KLH) *via* non-site-selective modification. There are still few examples of homogeneous glycovaccines,[266] though, due to the potential of this application, the obtained heterogeneous mixtures were already administrered to living organisms, which led to interesting vaccine candidates against alkaloid drug addiction and anti-cancer therapy.[14,267–272] These results attest the potential of this strategy and highlight the importance in developing new therapeutic approaches.

IV.1.2. Biomolecules' enhancement

Apart from the modifications made to mimic nature, more recently, addition of artificial agents to ease biomolecule purification, to modify its pharmacokinetics or even to confer previously absent therapeutic or imaging properties centred the attention of chemical modification of proteins. This enhancement allows the development of several applications that will help to solve some biological uncertainties. For instance, by performing certain modifications on specific proteins, the study of the protein's function and distribution *in vivo* is possible, as well as the development of effective drug-delivery systems, such as ADCs.[10,273]

IV.1.2.1. Bioimaging

Bioimaging is another common application of bioconjugation procedures. This technique allows an evaluation of the protein's function, interactions and distribution in biological assays and, ultimately, this system can be used as a diagnostic tool for certain pathologies.[30]

Several biomolecules were already labeled and applied as diagnostic tools, either by nuclear or by optical bioimaging methods. The difference between these two imaging approaches lies on the used imaging agent, which can be directly or indirectly incorporated through conjugation. Usually, the indirect incorporation of these molecules rely on the conjugation of chelating agents that can trap different radioisotopes with imaging properties (^{99}mTc, ^{18}F, ^{111}In, ^{123}I, ^{68}Ga). After chelation, a nuclear imaging method can be established, either by application of a positron emission tomography (PET), as much as a single photon emission computed tomography (SPECT). For optical fluorescence imaging, usually, molecules with imaging properties (like organic near-infrared fluorophores or quantum dots) are directly linked to the biomolecule.[151]

In terms of medical imaging, although nuclear imaging is still the first-line technique, optical imaging is expanding as a valuable alternative due to the similar sensitivity and greater spatial and temporal resolution, without resorting to radioactive metals.[50]

IV.1.2.2. Medicinal Chemistry

Chemical modification of biomolecules also allows the improvement of therapeutic biomolecules or organic molecules. By modifying therapeutic biomolecules, enhancement of their pharmacokinetic properties or efficacy on given pathologies can be achieved. Through modification of therapeutic organic molecules with biomolecules it is conceivable that one might introduce targeting moieties that would allow the development of drug-delivery systems. PEGylation of biomolecules and construction of drug conjugates with targeting biomolecules emerge as the most valuable and commonly used applications. In

this field, such strategies recently led to FDA-approved drugs, such as PEGylated forms of hormones and antibody-drug conjugates for anti-cancer therapy.[274]

Generally, PEGylation of therapeutic biomolecules increases *in vivo* stability, half-life, solubility, reduces toxicity, immunogenicity, antigenicity, kidney clearance and proteolysis. With such properties, this chemical modification of biomolecules improves pharmacokinetic parameters, such as the absorption rate and volume of distribution. This procedure has proven its utility in a large spectra of therapeutic applications, namely, different cancer types, acromegaly, hepatitis, gout or severe rheumatoid arthritis.[274] In order to highlight the relevance of the topic, BAY94-9027 (Figure **32**) can be mentioned as a PEGylated form of rFVIII that is currently on clinical trials to extend the circulating half-life of this transcript factor, while preserving its biological activity.[275]

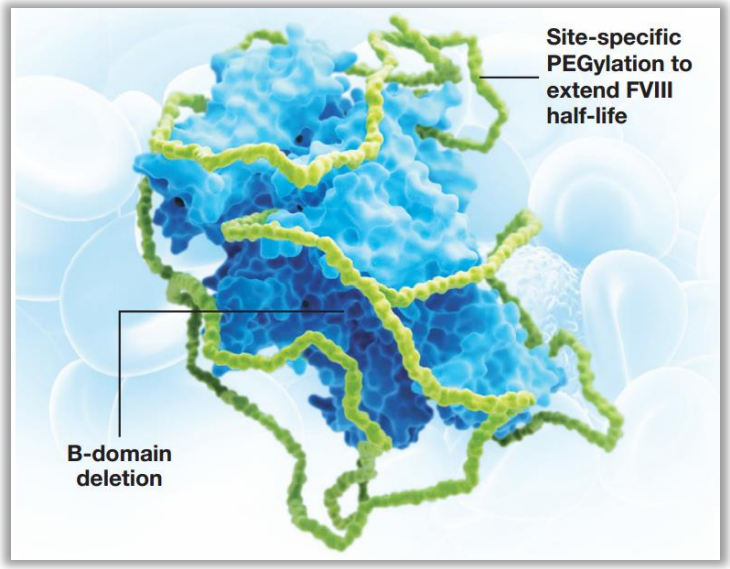


Figure **32** – Structure of BAY94-9027. Adapted from a poster of a conference from the European Directorate for the Quality of Medicines

Antibody-drug conjugates (ADCs) are one of the most promising biotech drugs, since that due to the unique targeting ability of antibodies, their attachment to therapeutic organic molecules, can lead to an effective therapy that spares healthy tissues by releasing its cargo only upon specific antigen recognition. However, despite ADCs' conceptual simplicity, their individual components must have precise properties to cause a therapeutic benefit. The antibody should have high affinity and specificity for a defined and abundant antigen and its pharmacokinetic properties should be unaffected upon conjugation with the drug. The therapeutic organic molecule should be highly potent, thereby minimizing the number of payload molecules necessary to induce a therapeutic effect. Finally, the conjugation strategy must permit chemical

installation of the drug onto the antibody at a pre-determined site(s), and ensure stability of the conjugate whilst in circulation *in vivo* (Figure **33**).

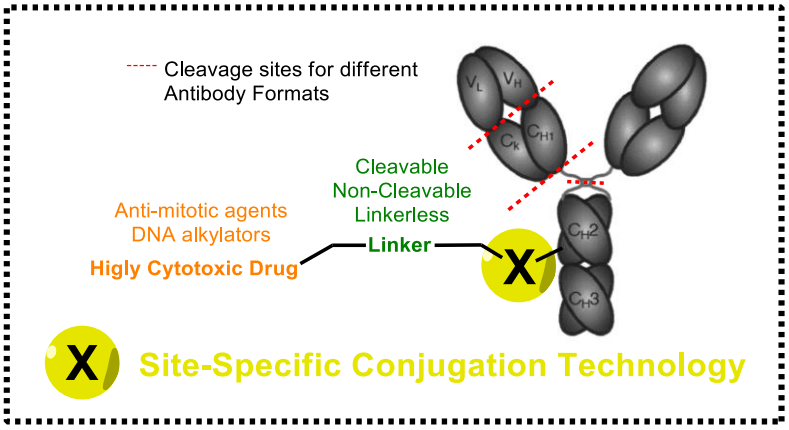


Figure **33** – ADC's general strutcture and features

Efforts in this field have led to the recent commercialization approval of two ADCs: Adcetris® (brentuximab vedotin), for the treatment of refractory Hodgkin lymphoma and anaplastic large-cell lymphoma, and Kadcyla® (ado-trastuzumab emtansine), for the treatment of metastatic Her2+ breast cancer (Figure **34**).[276] Though anti-cancer therapy has been the most widely application for ADCs, it is foreseeable that other pathologies can be valid targets to be pursued in a near future.

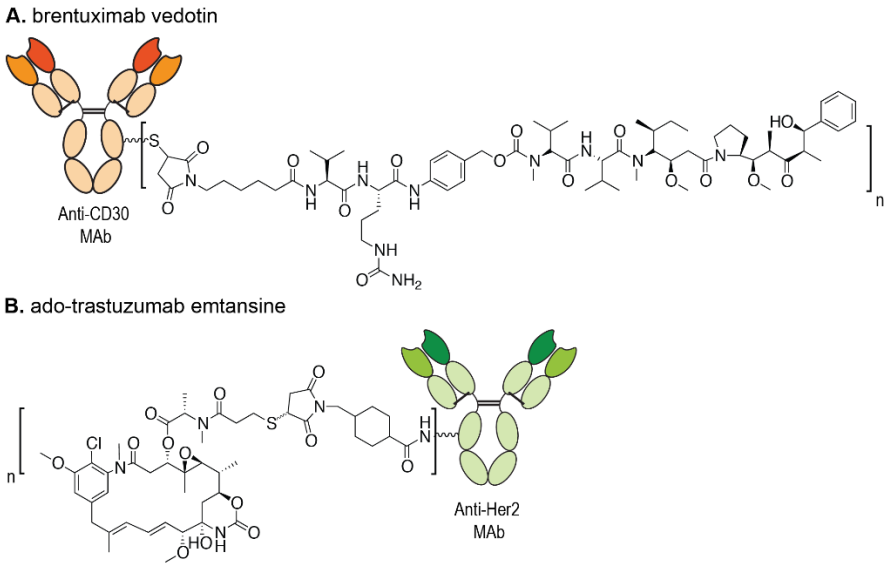


Figure **34** – Examples of recent FDA-approved ADCs: (A) Bretuximab vedotin – Adcetris® (assembled by means of maleimide conjugation); (B) Ado-trastuzumab emtansine – Kadcyla® (assembled by means of NHS conjugation).

Moreover, the targeting ability of antibodies is not exclusive of these biomolecules, once theoretically, any biomolecule that is selectively recognized by a receptor of a cell is a possible targeting moiety for a specific pathology. For instance, the vitamin folic acid (FA) displays high affinity towards a membrane protein (the folate receptor), that captures its ligands from the extracellular milieu and transports them inside the cell through an endocytic process. Since the expression of these receptors is largely absent from normal tissues, they are considered to be tumor antigens/biomarkers (Figure **35**).[40] Therefore, folate-conjugates are being used as alternatives to ADCs for anti-cancer therapeutics. Furthermore, as an additional information (though a little off-topic, since this methodology does not rely on any bioconjugation procedures), small organic molecules have also been, recently, identified as molecules capable of binding to certain biomolecules, therefore exhibiting a possibility of designing conjugates for therapeutic applications.[277,278]

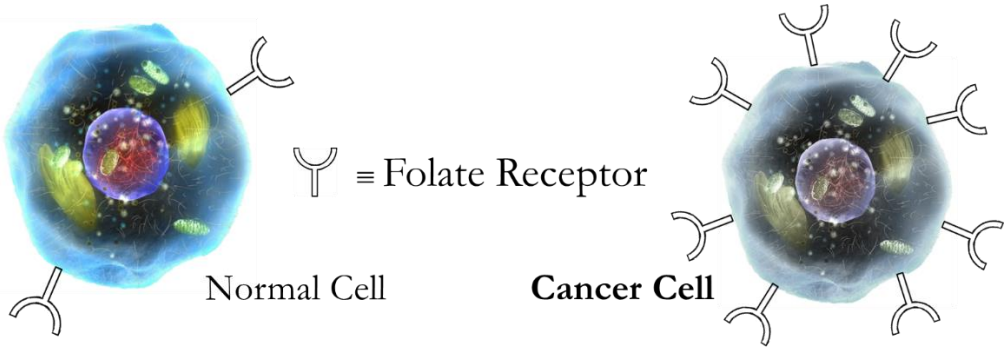


Figure **35** – Illustration of overexpression of folate receptors in cancer cells

IV.2. Goals

In this chapter, the development of 2-ABBA derivatives to functionalize biomolecules for a diverse set of applications will be presented.

These derivatives were prepared to perform an artificial glycosylation of proteins and a series of fluorescent 2-ABBA derivatives were also prepared and conjugated with different biomolecules, namely, with folate derivatives to study the potentially selective uptake by cancer cells.

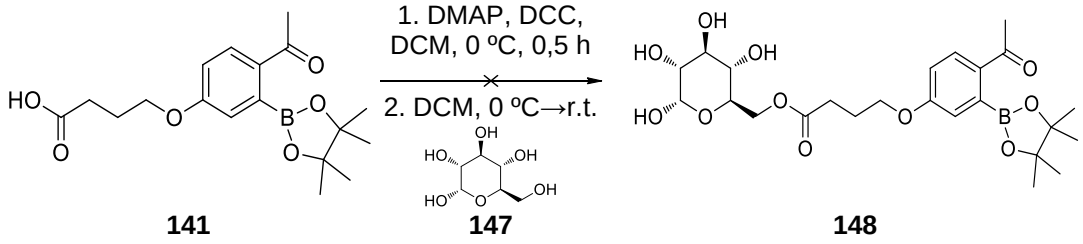
Finally, 2-ABBA derivatives were modified for therapeutic applications, namely to PEGylate peptides and proteins and to synthesize a folate-paclitaxel conjugate. The first method was used to study a sugar-dependent reversible PEGylation of insulin. The folate-paclitaxel conjugate was compared with the unconjugated cytotoxic drug in terms of toxicity and selectivity of internalization.

IV.3. Applications of modified 2-ABBA

In order to use this new bioconjugation methodology based on iminoboronate formation, it was necessary to functionalize the novel modifying agent presented in **Chapter III.** Therefore, compound **141** is always used as the starting point for all reported applications. Usually, in each application a convergent synthetic strategy was applied, in which the amidation/esterification reaction was the convergence point.

IV.3.1. Glycosylation

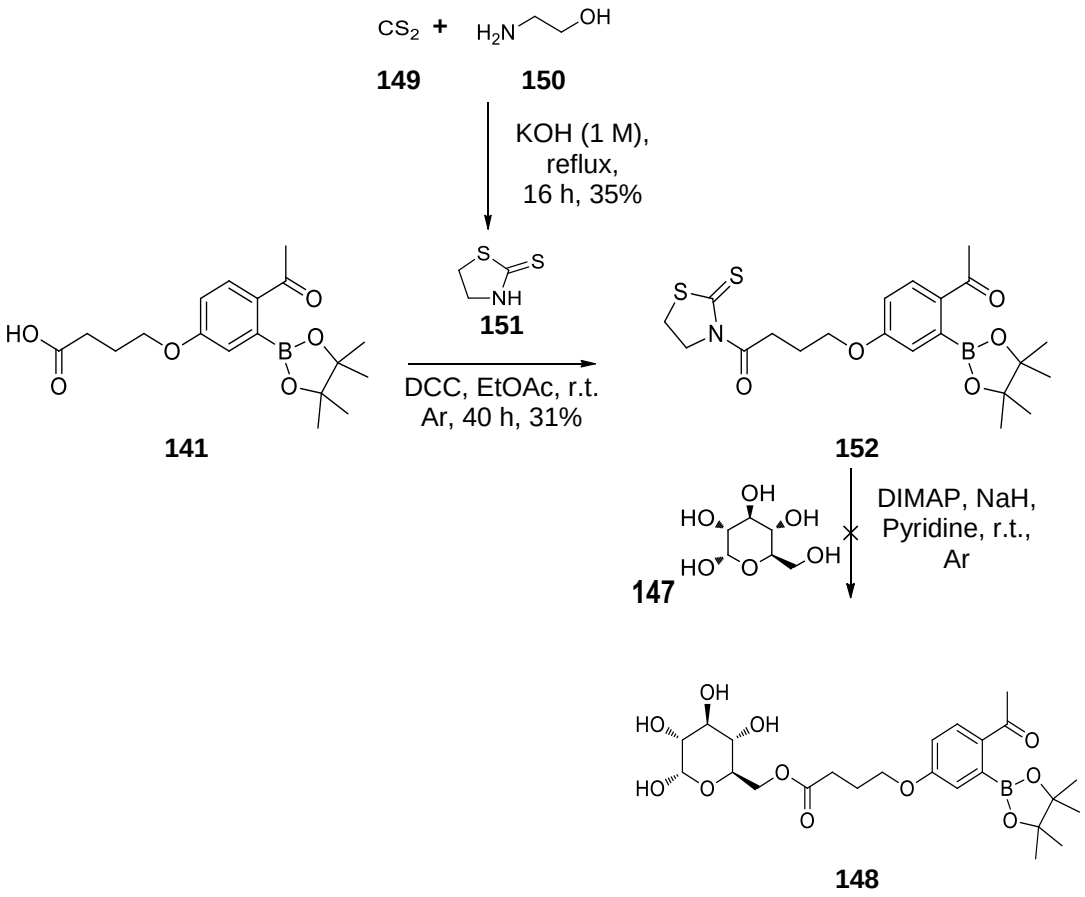
Having as a goal the incorporation of *α*-D-glucose into the structure of the novel modifying agent (**141**), first, a Steglish esterification was attempted, based on literature precedents that gave the primary alcohol of glucose as the best nucleophile of all the alcohols present in its structure (Scheme **54**).[279] We used compound **141** (pinacolyl boronate ester) instead of compound **140** (boronic acid) to avoid interactions between the sugar molecule and the boronic acid moiety. Unfortunately, no product was obtained and only derivatives of glucose without aromatic protons NMR signals were isolated.



141 **147** **148**

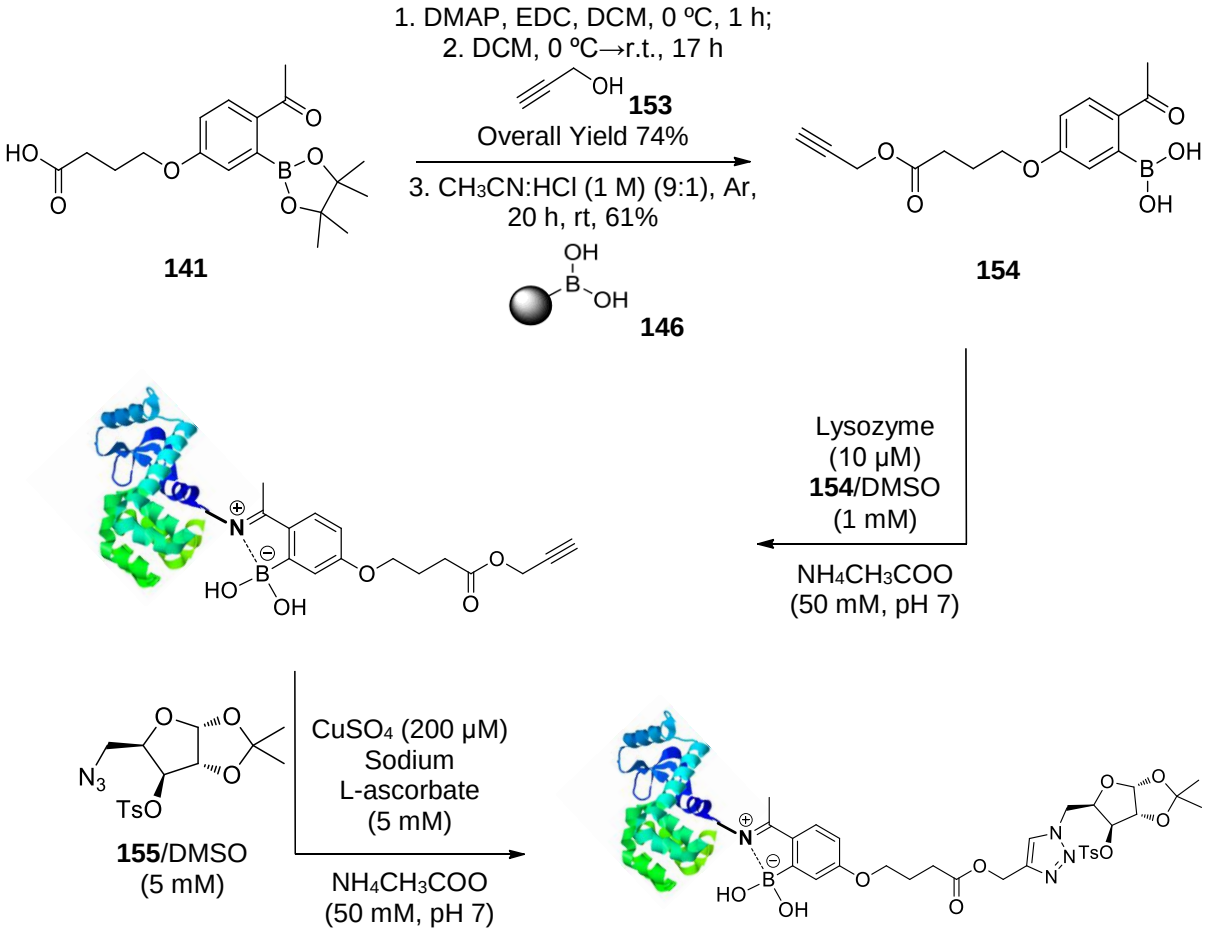
Scheme **54** – First attempt of constructing a glycosyl derivative of 2-ABBA

From this point, we have studied the hypothesis of having a poor activation of the carboxylic acid for the intended reaction by experimenting a different described protocol with a specific group to activate the carboxylic acid to perform sugar chemistry without protecting groups. In this case, a *N*-acylthiazolidine-2-thione intermediary (**152**) was isolated and this compound was reacted with unprotected *α*-D-glucose in order to obtain compound **148**. However, in this last step, the reaction afforded products with inconclusive characterization, that included possible dimerization products.



Scheme **55** – Second attempt of constructing a glycosyl derivative of 2-ABBA

Since this glycosylation was proving to be hard to obtain with unprotected saccharide molecules, we started to wonder if indirect coupling with a protected sugar molecule would not be a better strategy to afford the sought glycosyl derivative of 2-ABBA. Therefore, we outlined a synthetic route, in which there would be an indirect coupling through a click chemistry procedure (a CuAAC), after incorporating a bioorthogonal handle on compound **141**. Thus, a terminal alkyne derivative of 2-ABBA was synthesized (**154**) and tested as a modifying agent of lysine residues from lysozyme. From this point, a protected azidesugar (**155**) was used to perform a CuAAC on the protein's surface yielding the expected construct in ammonium acetate buffer (50 mM, pH 7.0) at room temperature as confirmed by ESI-FTICR-MS (Scheme **56**, *m/z* values of the lysozyme conjugates are described in **Chapter VI.**).



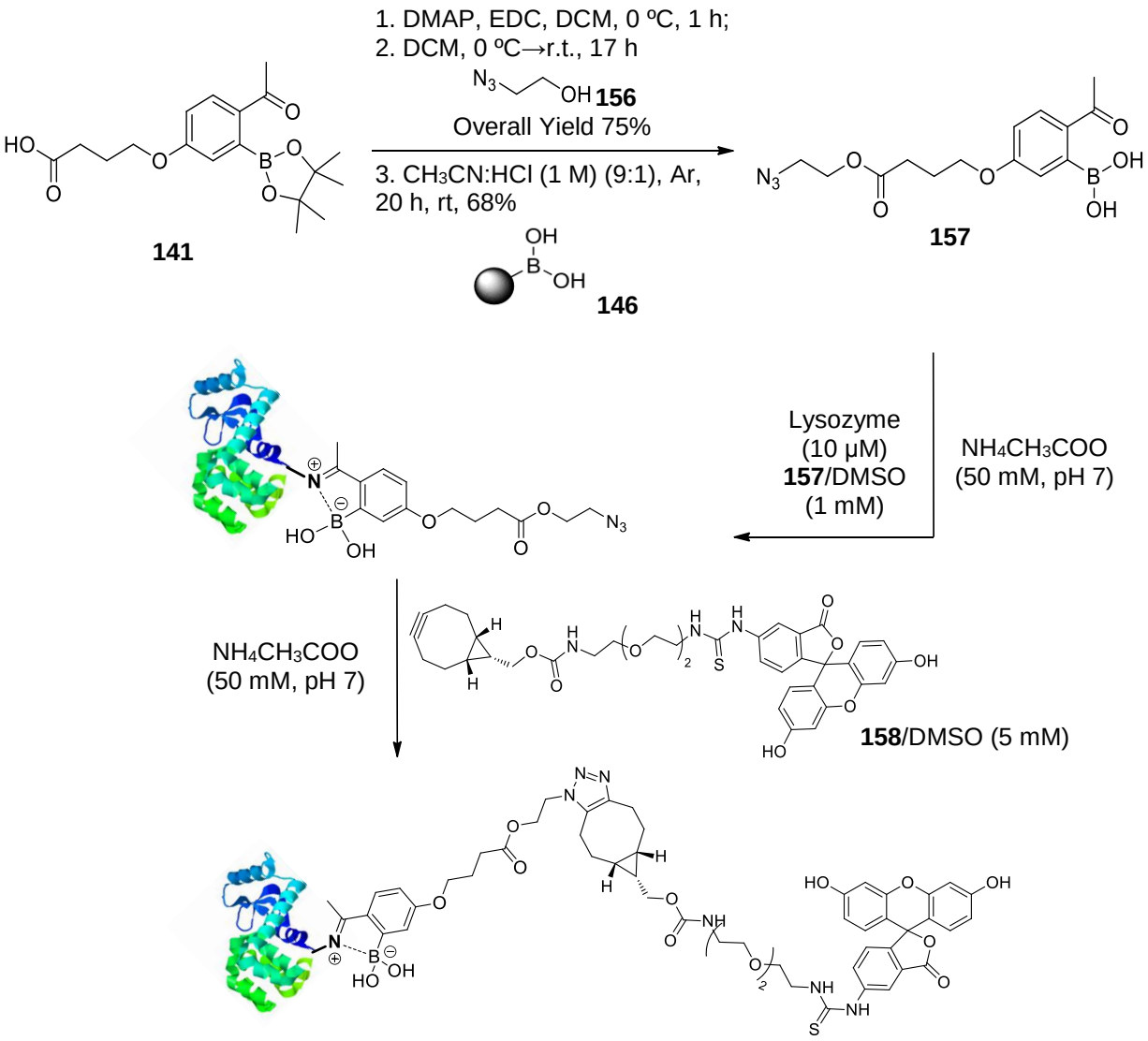
Scheme **56** – Third attempt of constructing a glycosyl derivative of 2-ABBA – Synthesis of the alkyne derivative of 2-ABBA (**154**) and reaction of lysozyme (10 μM) with compound **154** (1 mM) in ammonium acetate buffer (50 mM, pH 7) at room temperature. CuAAC between azidesugar (**155**) and the terminal alkyne on the protein's surface.

Therefore, this application demonstrated the feasibility of using this iminoboronate methodology and combine it with the first bioorthogonal reaction. Moreover, from this specific reaction, finally, the first glycosyl derivative conjugate was obtained, which means that an artificial glysocylation of lysozyme was completed.

IV.3.2. Bioimaging

Led by the success of the previous application, once the click chemistry was applied to indirectly attach a glycosyl derivative to the core molecule of the modifying agent, a new strategy was envisioned where another click chemistry procedure (SPAAC) would be used to attach a fluorescent molecule. Based on those premises, a different derivative of 2-ABBA bearing a bioorthogonal handle (an azide) was synthesized (**157**) and readily tested as a modifying agent of lysine residues from lysozyme. From that

point, having the azide moiety indirectly installed in a protein a SPAAC with a cyclooctyne-fluorescein derivative (**158**) was performed on the protein's surface yielding the expected constructs in ammonium acetate buffer (50 mM, pH 7.0) at room temperature as confirmed by ESI-FTICR-MS (Scheme **57**, *m/z* values of the lysozyme conjugates are described in **Chapter VI.**).

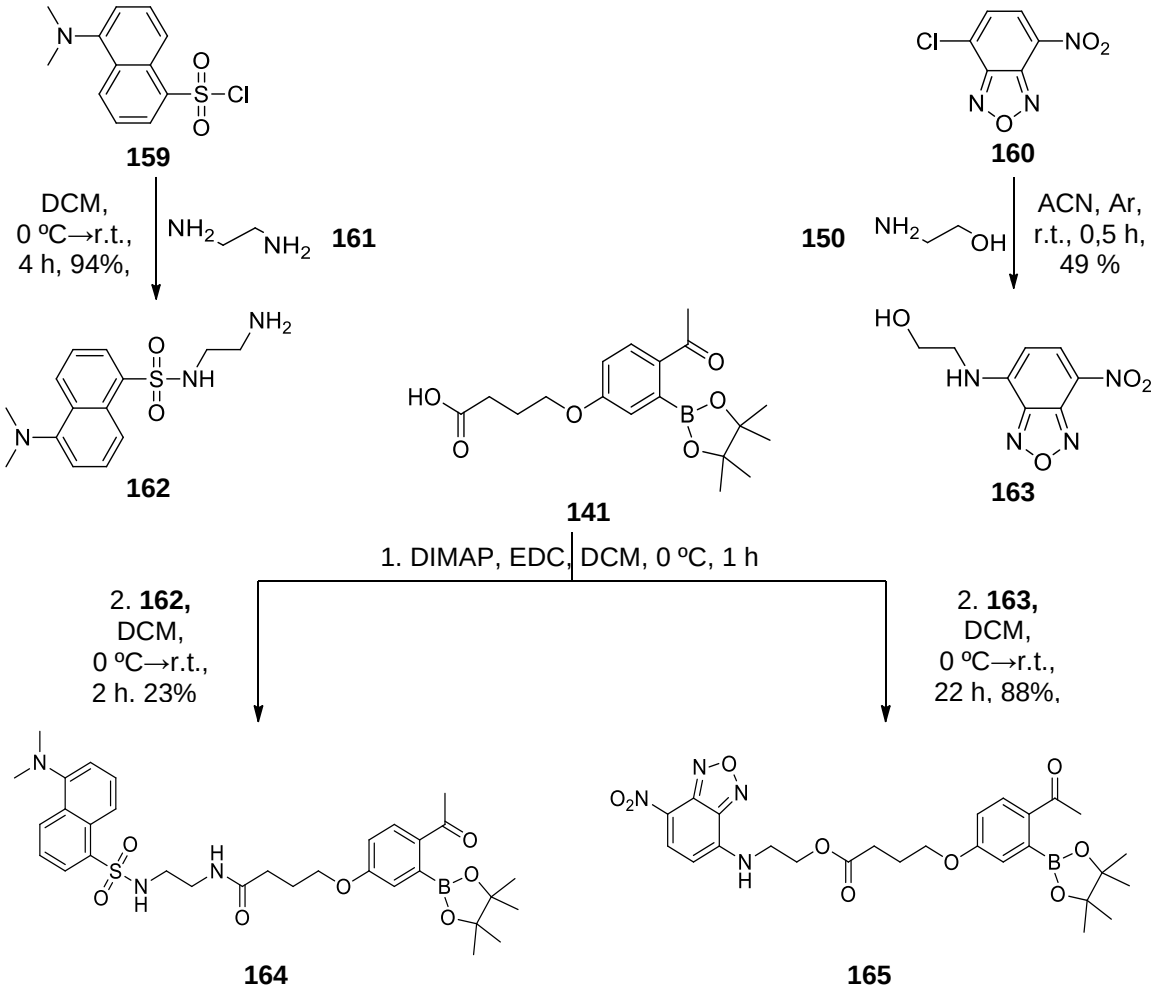


Scheme **57** – Synthesis of the azide derivative of 2-ABBA (**157**) and reaction of lysozyme (10 μM) with compound **157** (1 mM) in ammonium acetate buffer (50 mM, pH 7) at room temperature. SPAAC between cyclooctyne-fluorescein derivative (**158**) and the azide on the protein's surface.

Therefore, this application demonstrated the feasibility of using this iminoboronate methodology and combine it with another bioorthogonal reaction. Moreover, from this specific reaction, the first conjugate bearing a fluorescent probe was synthesized.

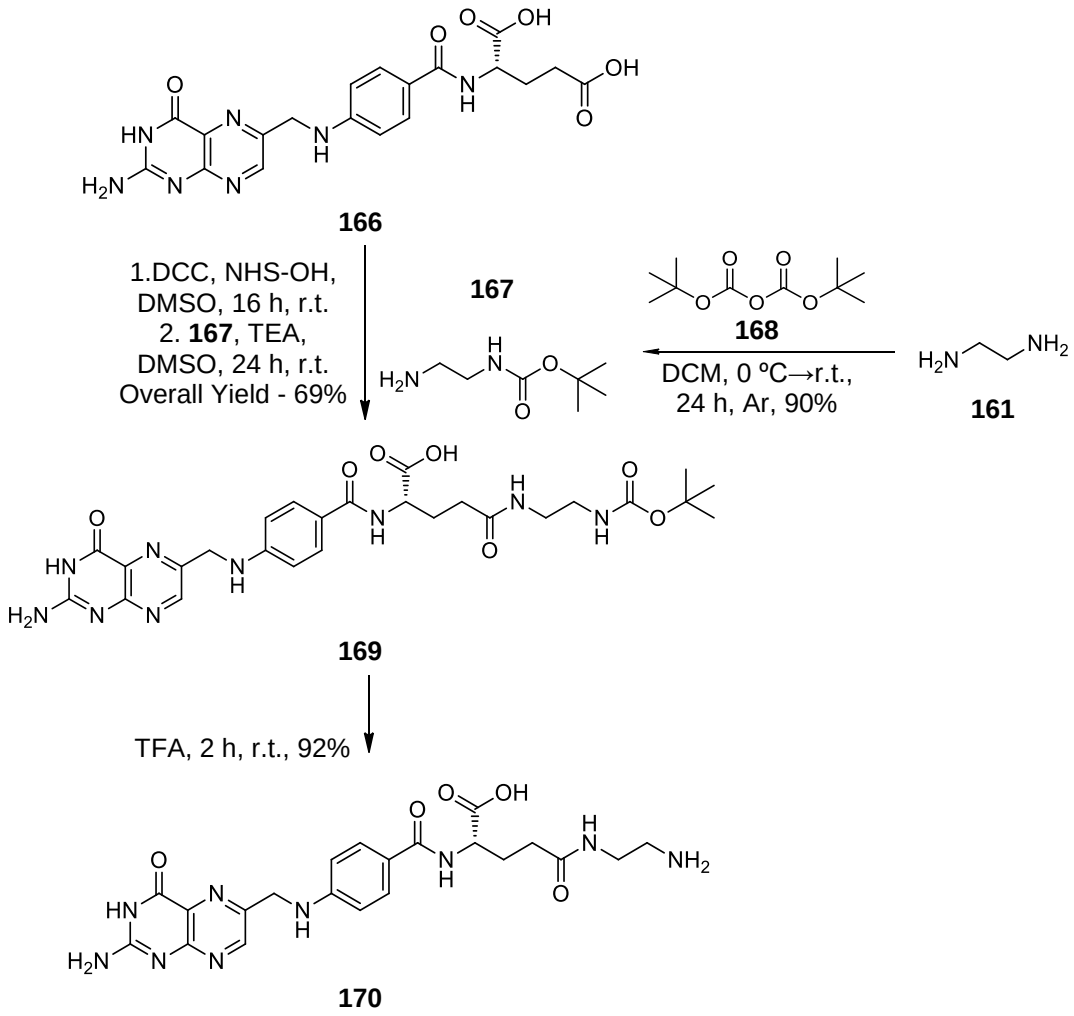
To test the possibility of using iminoboronate methodology in biological assays, a demonstration that the N-B interaction could endure cellular internalization had to be done. Thus, we next evaluated the possibility of designing a cancer cell targeting conjugate, in which, a fluorescent motif was linked to a targeting molecule through an iminoboronate conjugation. This was achieved by synthesizing fluorescent 2-ABBA derivatives and conjugate them with *N*-(2-aminoethyl) folic acid (EDA-FA), which, in turn generated conjugates that were selectively recognized and internalized by cancer cells.

Dansyl and nitrobenzofurazan fluorescent motifs were modified with ethylenediamine and ethanolamine, respectively, and each subsequently reacted with **141** (Scheme **58**). Thus, two fluorescent derivatives of 2-ABBA were synthesized, though a deprotection step (removal of pinacolyl group) would still be required before performing the conjugation.



Scheme **58** – Synthetic routes to obtain two fluorescent derivatives of 2-ABBA

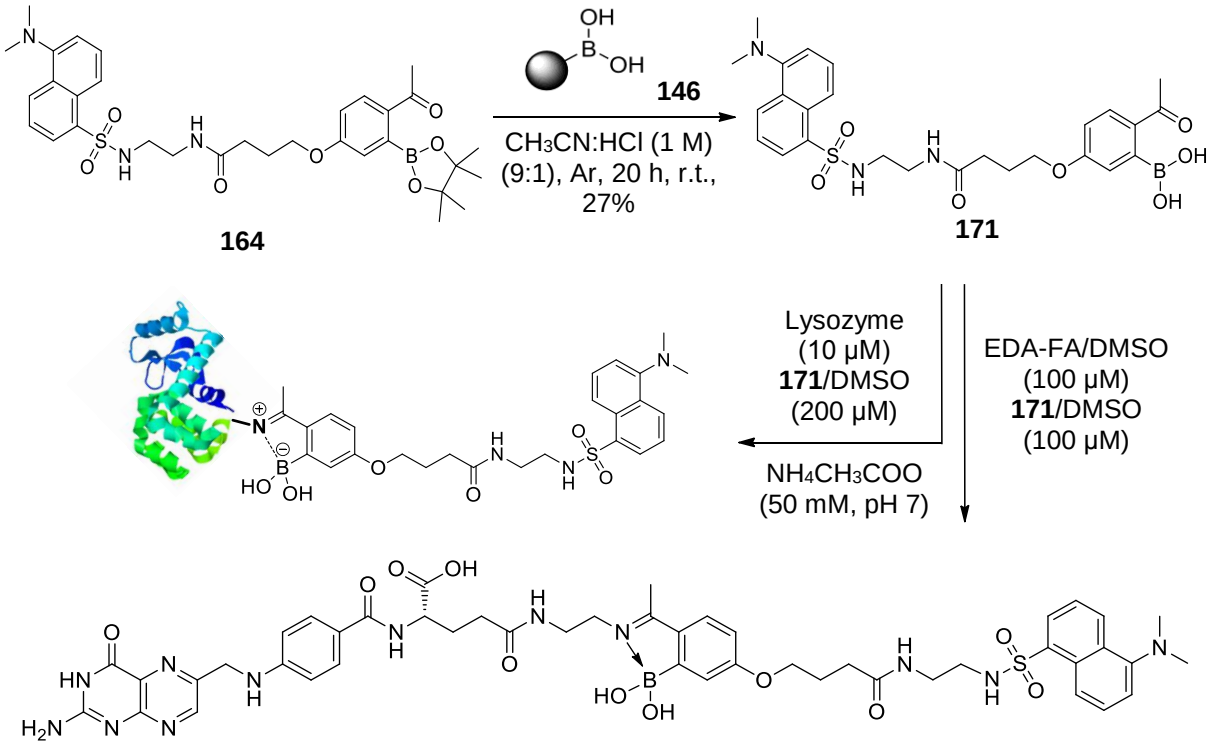
As it was mentioned in the introduction, folate receptors are over-expressed in cancer cell lines due to their fast cell division and growth. Therefore, folic acid has been extensively used as a recognition moiety in several conjugates. Bearing that in mind, another key compound to advance with this strategy is the synthesis of a folic acid derivative, since it requires an available alkylic primary amine to form the intended iminoboronate conjugates. Thus, based on reported literature to synthesize *N*-(2-aminoethyl) folic acid (**170**, EDA-FA), this derivative was synthesized and stored.



Scheme **59** – Synthesis of *N*-(2-aminoethyl) folic acid (**170**, EDA-FA)

Next, in order to be able to generate iminoboronates, removal of the pinacolyl protecting group was essential. A transesterification method that uses a boronic acid resin (**146**) under acidic conditions was used as the deprotecting procedure that afforded the final fluorescent compounds **171** and **173** (Scheme **60** and Scheme **61**). Once prepared, compounds **171** and **173** were immediately tested for their ability to conjugate with lysozyme. Gratifyingly, both compounds retained their ability to functionalize the protein,

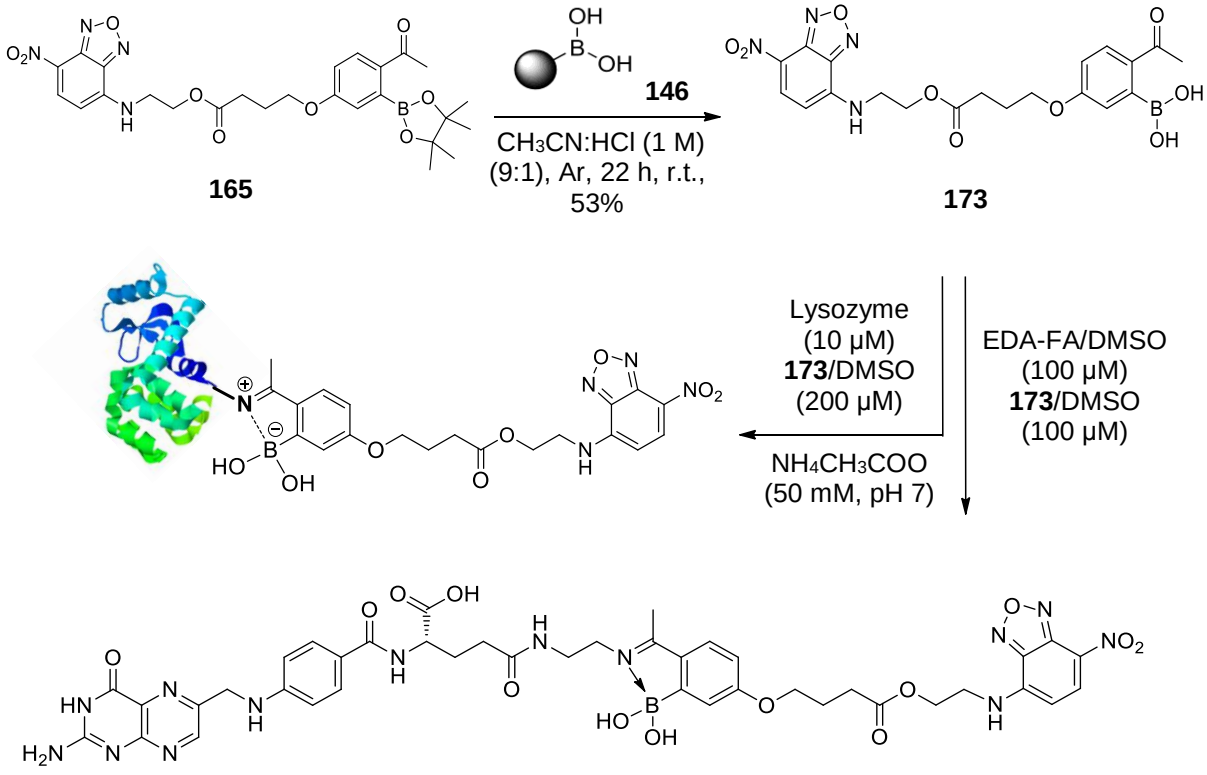
yielding the expected constructs in ammonium acetate buffer (50 mM, pH 7.0) at room temperature as confirmed by ESI-FTICR-MS (*m/z* values of the lysozyme conjugates are described in **Chapter VI.**). Encouraged by these results, we next evaluated the possibility of designing a cancer cell targeting conjugate, in which, the fluorescent motif is linked to the targeting molecule by a N-B bond.



ESI-MS: *m/z* $[M+H-H_2O]^+_{calculated}$ = 989.39022, *m/z* $[M+H-H_2O]^+_{real}$ = 989.39107

Scheme **60** – Deprotection of pinacolyl boronate ester to generate **171**. Reaction of lysozyme (10 μM) with compound **171** (200 μM) in ammonium acetate buffer (50 mM, pH 7) at room temperature. Reaction of EDA-FA (100 μM) with compound **171** (100 μM) in the same buffer to obtain **172** confirmed by HRMS.

Therefore, the conjugation of **171** and **173** with EDA-FA was evaluated. As expected, upon the addition of 100 μM of **171** or **173** in DMSO to EDA-FA in ammonium acetate buffer (50 mM, pH 7.0) at room temperature, their corresponding iminoboronates were readily formed, as characterized by high resolution mass spectrometry – HRMS (Scheme **60** and Scheme **61**). Following this, the conjugates' ability to differentiate cancer and non-cancer cell lines was then studied.



ESI-MS: *m/z* $[M+H]^+_{calculated}$ = 938.33545, *m/z* $[M+H]^+_{real}$ = 938.33917

Scheme **61** – Deprotection of pinacolyl boronate ester to generate **173.** Reaction of lysozyme (10 μM) with compound **173** (200 μM) in ammonium acetate buffer (50 mM, pH 7) at room temperature. Reaction of EDA-FA (100 μM) with compound **173** (100 μM) in the same buffer to obtain **174** confirmed by HRMS.

To study the internalization of fluorescent conjugates **172** and **174**, conjugate **172** was generated prior to exposure to cells and tested against human non-small lung cancer cells (NCI-H460) and non-cancer human kidney embryonic cells (HEK 293). Interestingly, laser scanning confocal images of the HEK 293 and NCI-H460 treated with **172**, revealed that only the cancer cell line that over-expressed the FA receptor up took fluorescence, which suggests selective folate-receptor mediated internalization (Figure **36**).

To further elucidate this mechanism, the cancer cell line NCI-H460 was treated with **173** without EDA-FA. In agreement with a folate-receptor mediated internalization mechanism, without the recognition moiety, no fluorescence was detected inside the cells (Figure **37**C). In contrast, when the same compound (**173**) was allowed to form the conjugate with EDA-FA, the previously generated N-B construct **174** smoothly underwent internalization as shown in Figure **37**D. Finally, the NCI-H460 cells were treated with EDA-FA prior to the addition of the conjugate **174**. In this case, the internalization of the conjugate **174** was considerably impaired due to the initial exposure to folic acid that reduced the cell's need for this vitamin (Figure **37**E). These results clearly stress the importance of the folate moiety to mediate the internalization process.

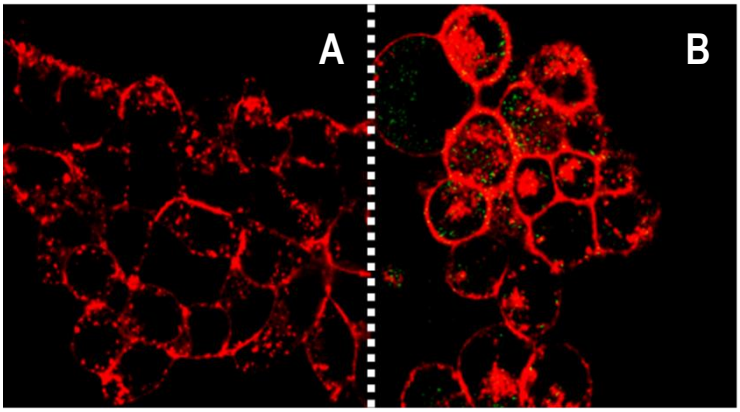


Figure **36** – HEK (A) and NCI-H460 (B) cells were incubated with compound **172** at 20 μM for 4 h. Images are an overlay of the emission of compound **172** in the 460-580 nm region upon excitation at 458 nm (green), and the emission of the membrane marker in the 650-700 nm region upon excitation at 514 nm (red).

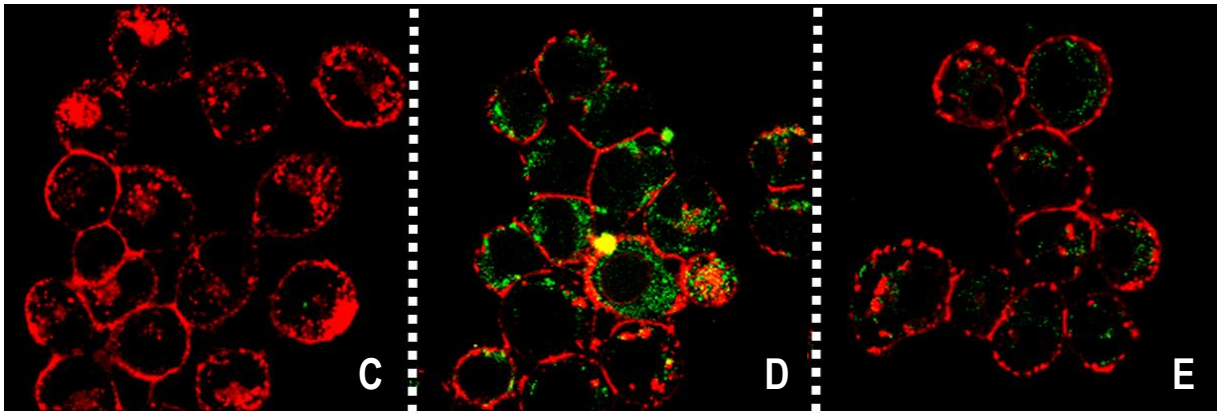
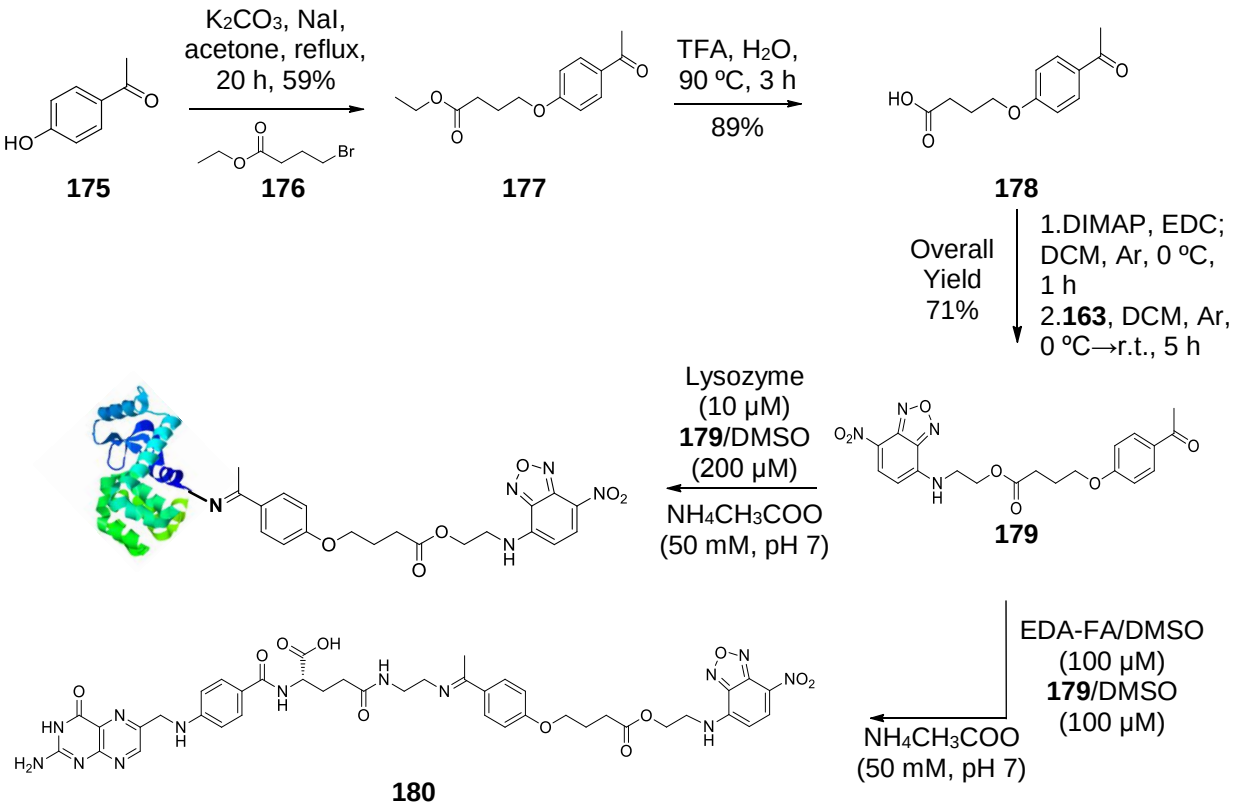


Figure **37** – NCI-H460 (C) was incubated with compound **173**, NCI-H460 without (D) and with pre-treatment with EDA-FA (E), were incubated with compound **174**. Incubation and imaging conditions are the same as described in Figure **36**.

Finally, to determine the contribution of the N-B bond to the formation and stability of these conjugates, the fluorescent compound **179**, featuring no boronic acid was prepared *via* alkylation of 4-hydroxyacetophenone with ethyl 4-bromobutyrate, followed by hydrolysis and *N,N*-dimethylpyridin-4-amine (DMAP) catalysed esterification with 4-ethanolamine-7-nitrobenzofurazan (**163**). Once prepared, compound **179** was solubilised in DMSO and combined with EDA-FA in ammonium acetate buffer solution (50 mM, pH 7.0) at room temperature to form conjugate **180**, as confirmed by HRMS. A mixture containing the conjugate was then used to treat the NCI-H460 cancer cells. This resulted in no fluorescence being detected inside the cells, presumably due to the poor stability of conjugate **180** under these conditions.

Therefore, we studied the ability of compound **179** to modify lysozyme. The compound lacking the boronic acid functionality hardly formed the expected constructs with the protein after 30 min in ammonium acetate buffer (50 mM, pH 7.0). Even the ones that did form, seemed to hydrolyse in only 2 h under these

reaction conditions. In stark contrast, in the same conditions, boronated compound **173** readily afforded the constructs with lysozyme within 30 min and the modifications were persistent after 2 h in ammonium acetate buffer (50 mM, pH 7.0) at room temperature. These results clearly highlight the contribution of a boronic acid to imine stabilization and internalization of the conjugates.



ESI-MS: *m/z* $[M+H]^+_{calculated}$ = 894.32796, *m/z* $[M+H]^+_{real}$ = 894.32778

Scheme **62** – Synthetic route to obtain the aboronated species (**179**). Reaction of lysozyme (10 µM) with **179** (200 µM) in ammonium acetate buffer (50 mM, pH 7) at room temperature. Reaction of EDA-FA (100 µM) with compound **179** (100 µM) in ammonium acetate buffer (50 mM, pH 7) to obtain **180** confirmed by HRMS.

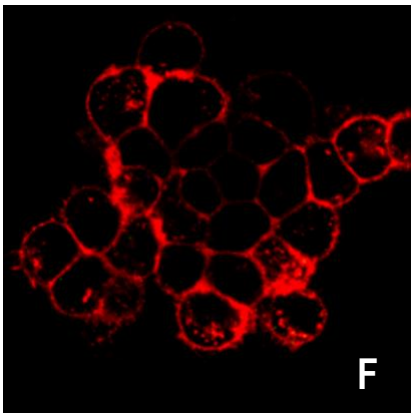
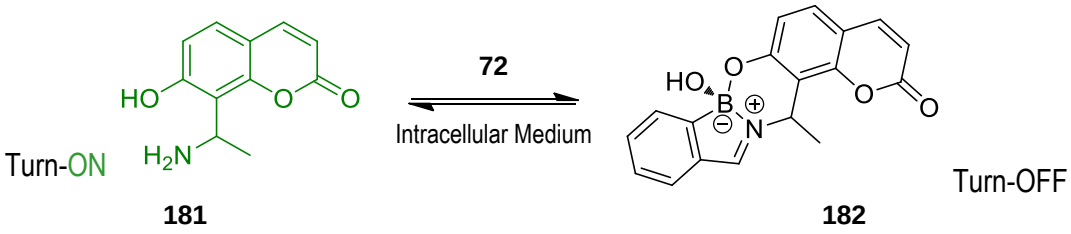


Figure **38** – Image F shows NCI-H460 treated with compound **180**. Incubation and imaging conditions are the same as described in Figure **36**.

Therefore, this work showed for the first time that the N-B bond may be used to synthetize conjugates that selectively target cancer cells. Fluorescent 2-ABBA derivatives were successfully prepared and conjugated *via* a N-B linkage with lysozyme and N-(2-aminoethyl) folic acid, generating conjugates that were selectively recognized and internalized by NCI-H460 cancer cells, which over-express folic acid receptors. The ability of these iminoboronates to undergo a receptor mediated internalization and their efficiency to promote the selective and reversible functionalization of proteins, highlights these constructs to have a promising future in the design of conjugates that selectively target cancer cells.

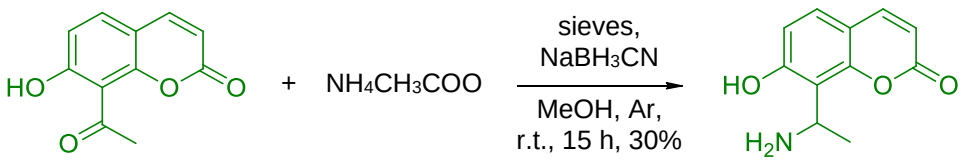
Though the internalization of conjugates bearing a N-B interaction was proven by the previous study, the possibility of releasing its cargo intracellularly was still unproven. Therefore, a strategy to develop an "ON-OFF" probe was envisioned for iminoboronates, where a coumarin would act as the fluorescent probe that would only be fluorescent if not conjugated to 2-carbonylbenzeneboronic acids (Scheme **63**).



181 **182**

Scheme **63** – Envisioned "ON-OFF" probe and its interaction with 2-carbonylbenzeneboronic acids

Based on fact that the fluorescence of 7-hydroxycoumarin dyes can be decreased by disguising the 7-phenolic hydroxyl group,[280] we rationally designed compound **181** to complex with 2-carbonylbenzeneboronic acid derivatives in order to have a turn-OFF probe whenever the iminoboronate is formed. This is an on-going study and so far only preliminary data can be reported. First, the synthesis of **181** is being optimized (Scheme **64**), though the complexation with iminoboronates is not well characterize yet. After a proper study of the fluorescent properties of such probe and its complexation product is finished, biological *in vitro* assays will be conducted in order to determine whether or not the cargo is released inside the cell.



183 **184** **181**

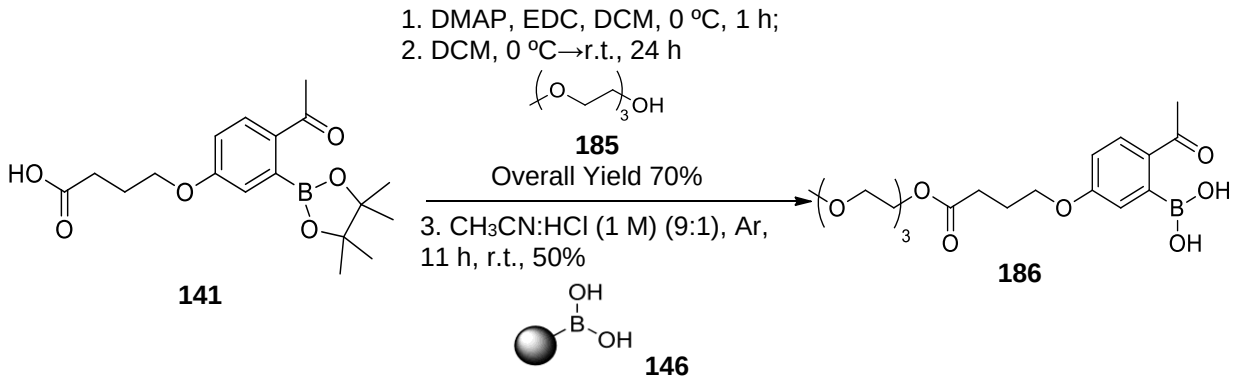
Scheme **64** – Synthesis of the fluorescent probe **181**

Though the bioimaging results of this project are not fully complete yet, this iminoboronate bioconjugation has proven its value at being stable enough to be internalized by cancer cells. Therefore, is plausible to assume that such bioconjugation methodology can be used to develop therapeutic applications that can endure biological assays.

IV.3.3. Therapeutics

Once the applicability of iminoboronate methodology in therapeutic applications was proved to be a possible development of such bioconjugation procedure, two strategies with therapeutical potential were envisioned. One based on the enhancement of pharmacokinetic properties of biomolecules and another that intends to improve the efficacy/selectivity of a certain drug through targeted delivery.

Starting from the bioconjugation methodology that could enhance the characteristics of a biomolecule through a PEGylation procedure, we have started by synthesizing a PEG derivative of 2-ABBA. To do so, compound **141** was modified through a Steglish esterification with a commercially available polyethylene glycol (**185**), affording the product in 70% yield. Next, the pinacolyl deprotection step afforded compound **186** in 50% yield (Scheme **65**). Once prepared, this compound was immediately tested for its ability to conjugate with lysozyme and, pleasantly, it was able to functionalize the protein, yielding the expected constructs in ammonium acetate buffer (50 mM, pH 7.0) at room temperature as confirmed by ESI-FTICR-MS (Figure **39**).



Scheme **65** – Synthesis of a 2-ABBA PEG derivative

When this molecule was fully characterized and its ability to modify primary amines tested, the following step was to modify therapeutic peptides in order to have a possibility of enhancing their pharmacokinetic properties. Peptide/protein hormones can be therapeutic entities with suboptimal

pharmacokinetic properties and, therefore, they are usual targets of several bioconjugation strategies. Somatostatin, lanreotide (a pharmacophore of somatostatin)[281] and insulin[282] are examples of these biomolecules with different therapetutic indications. Though the biological assays to prove the advantage of having PEGylated therapeutic peptides using iminoboronate methodolgy were not performed, successful PEGylation of somatostatin, lanreotide and insulin were achieved (Figure **39**).

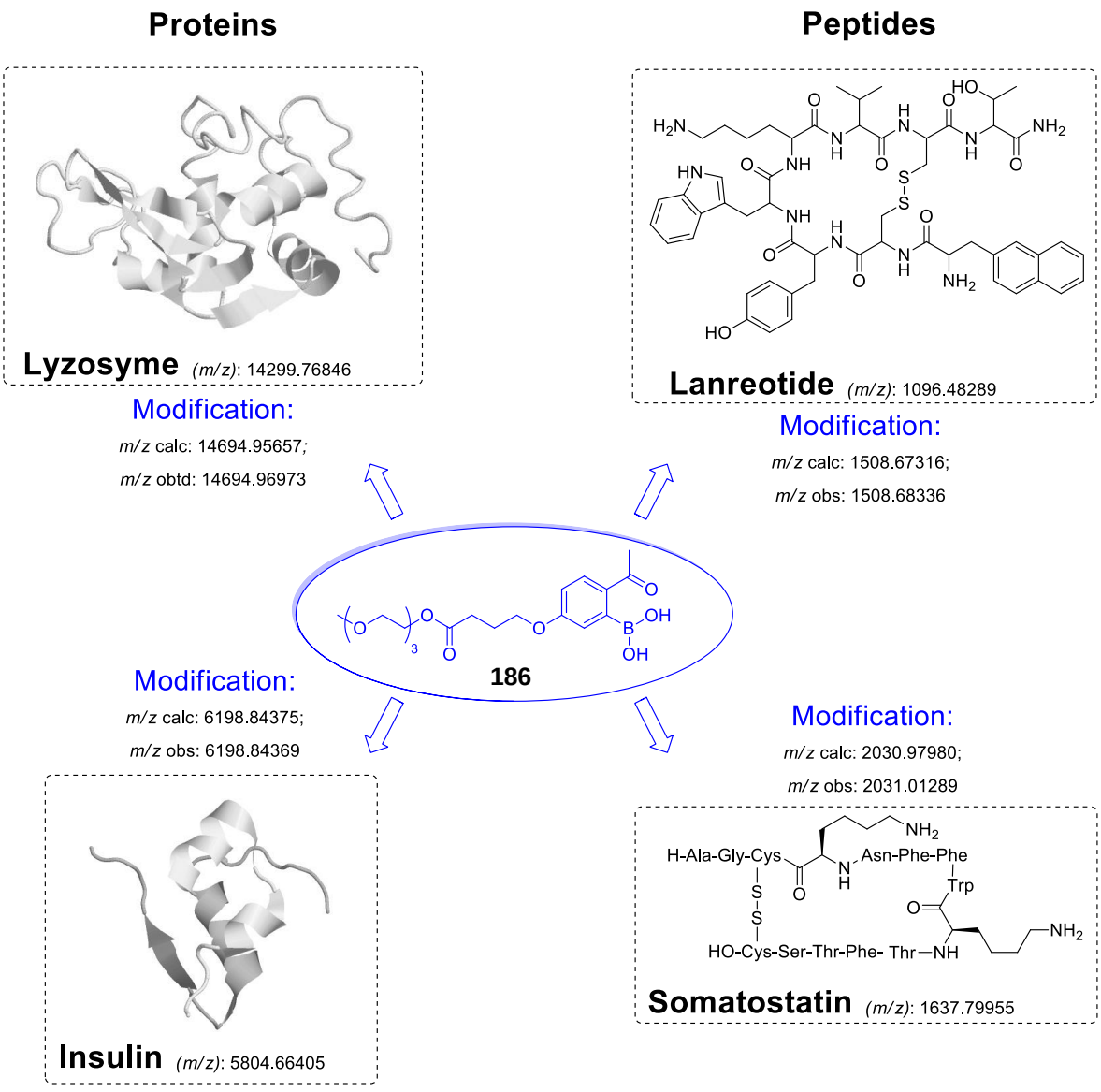


Figure **39** – Protein and Peptide Modifications with the PEG derivative of 2-ABBA (**186**)

Furthermore, taking into consideration that there is still a demand for improved insulin formulations that mimic the physiological profile after endogenous insulin secretion, insulin-conjugates have been widely tested to improve insulin's pharmacokinetics.[282] Since, unlike pathological situations, the

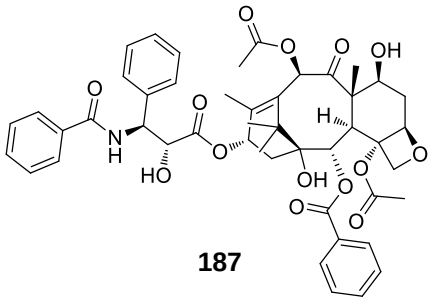
physiological levels of insulin are elevated when in presence of high blood sugar levels, a strategy that stabilizes insulin in circulation and releases its active form when the concentration of saccharide molecules is high is quite interesting.

Due to the potential reversibility of iminoboronate methodology with saccharide molecules, the ability of fructose to release this insulin's PEGylation was evaluated by mass spectrometry. This procedure was based on the construction of insulin-iminoboronate conjugates in ammonium acetate buffer (50 mM, pH 7.0) at room temperature, followed, by the addition of fructose and direct evaluation of the reaction mixture, through ESI-FTICR-MS (Table **12**). By mass spectra analysis, it was concluded that the presence of high concentrations of fructose disrupts the N-B interaction causing the release of free insulin. Therefore, this iminoboronate methodology can be interesting to improve insulin's properties.

Table **12** – Reversibility of Insulin's PEGylation through addition of Fructose

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Insulin	5804.66358	$[M+H]^+$	100.0	-
Insulin + ***186***	6198.84587	$[M+H]^+$	40.6	6198.85059
Insulin + 2x***186***	6593.01857	$[M+H]^+$	23.7	6593.03029
Insulin + 3x***186***	6987.18817	$[M+H]^+$	10.2	6987.20999
Insulin + ***186*** + *Fructose*	6198.854375	$[M+H]^+$	15.1	6198.851333
Insulin + 2x***186*** + *Fructose*	6593.023645	$[M+H]^+$	9.8	6593.031036
Insulin + 3x***186*** + *Fructose*	6987.194475	$[M+H]^+$	5.1	6987.210739

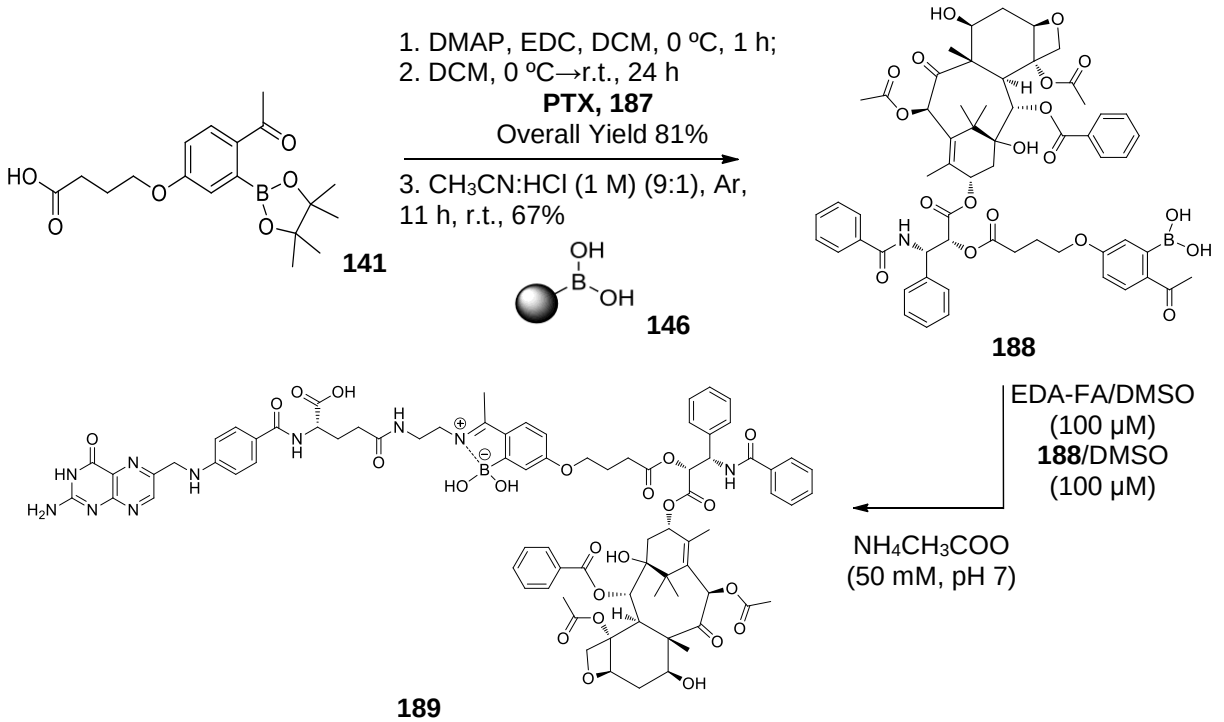
The second therapeutic application envisioned for iminoboronate methodology was based on a targeted drug-delivery system based on folate-conjugates. For this a derivatization of 2-ABBA core molecule was performed to introduce a cytotoxic drug. Paclitaxel (PTX, **187**) is an antimitotic agent that stabilizes altered microtubule strutures, thus disabling its normal degradation during cell division. This taxoid is especially cytotoxic to cancer cells, since these divide faster than other cells, though intestinal, hair follicles and myeloid cells can also be destroyed during this process, resulting on the known side effects of chemotherapy, like nausea, hair loss and myelosuppression.[39] Therefore, finding new and safer avenues to deliver this potent cytotoxic drug is of crucial importance and that is the main objective of this second therapeutic application.



187

Figure **40** – Paclitaxel's structure

Folate-iminoboronate conjugates were chosen as the constructs able to carry PTX to cancer cells. A synthetic pathway to generate a 2-ABBA PTX derivative is depicted in Scheme **66**. Starting from the acid derivative of the novel modifying agent (**141**), a Steglish esterification was performed, affording the product in 81% yield, followed by the already mentioned pinacolyl deprotection step that afforded compound **188** in 67% yield. Once this compound was synthesized, its ability to conjugate with primary amines was evaluated by HRMS, either with 1-butylamine or with the same derivative of folic acid used during the bioimaging studies (EDA-FA).



ESI-MS: *m/z* $[M+H]^+_{calculated}$ = 1567.62499,
m/z $[M+H]^+_{real}$ = 1567.62473

Scheme **66** – Synthesis of a 2-ABBA PTX derivative and reaction of EDA-FA (100 µM) with compound **188** (100 µM) in ammonium acetate buffer (50 mM, pH 7) to obtain **189** confirmed by HRMS.

Once the ability of this 2-ABBA derivative to conjugate with primary amines was shown to be unaffected by the large size of PTX, the targeted delivery of compound **189** was tested in cancer cell lines. Unfortunately, the IC_{50} obtained for this bioconjugate was higher than PTX alone (21 nM for the conjugate versus 6 nM for PTX), meaning that this conjugate has proven to be less potent.

The prepared bioconjugates and compounds were incubated with NCI-H460 cell line and the anti-proliferative assays performed. As depicted in Figure **41**, conjugate **189** was evaluated based on three control assays, which were made with: a) PTX (**187**) alone, b) PTX 2-ABBA derivative (**188**) alone and c) the same compound (**188**) conjugated with 1-butylamine (as a model iminoboronate that does not possess a targeting moiety). Based on the IC_{50} values determined, it was concluded that the conjugate under evaluation (**189**) has a better anti-proliferative effect than the PTX 2-ABBA derivative and the 1-butylamine iminoboronate conjugate, however conjugate **189** was less potent than PTX (**187**).

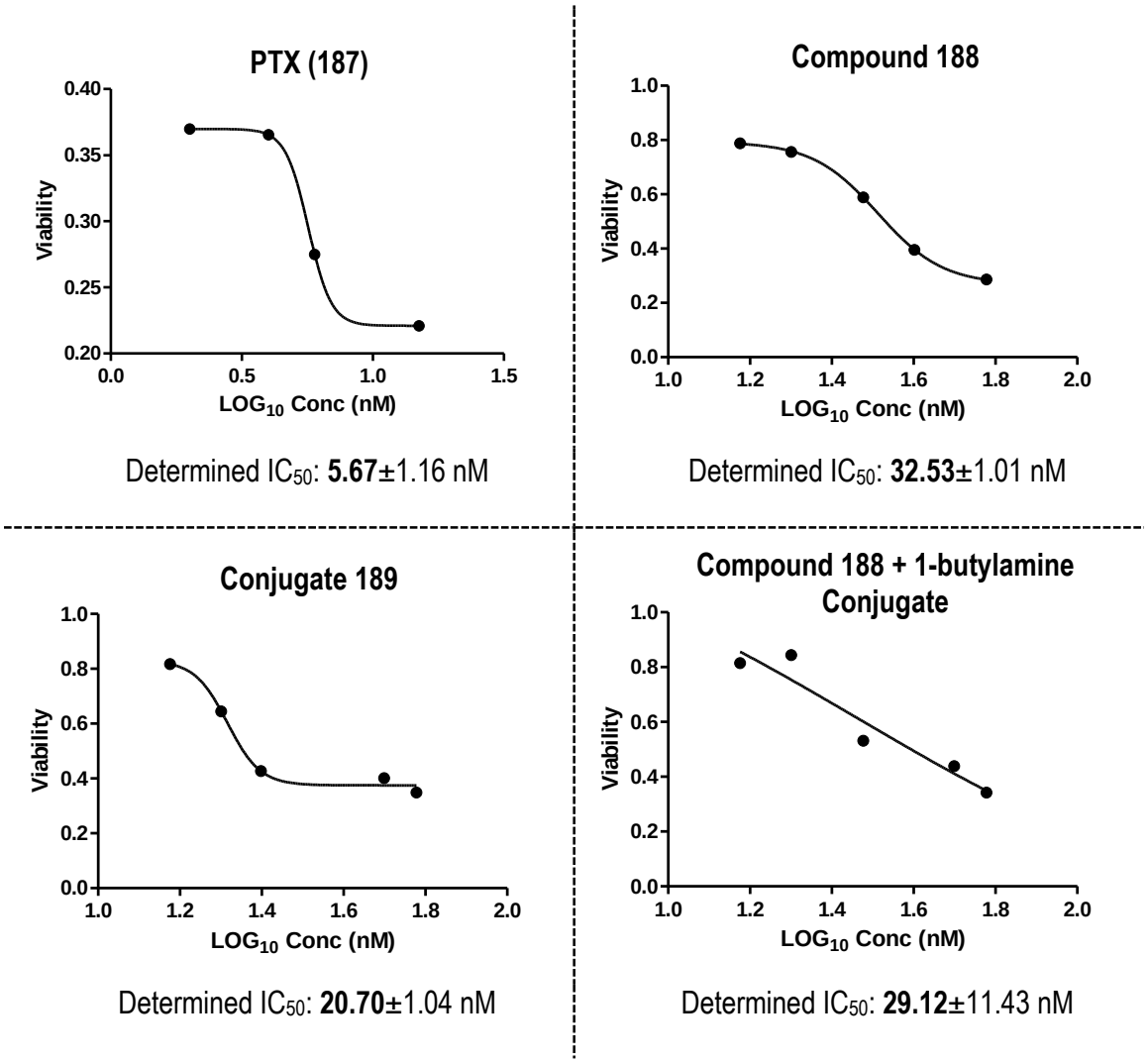


Figure **41** - Toxicity curves obtained for the NCI-H460 cell model and the determined IC_{50} – 48 hs of incubation

These results highlight that the targeted therapy is advantageous for any of the PTX derivatives synthesized (either bearing a ketone or an iminoboronate moiety), though the unmodified PTX appears to be more active. The fact that the conjugate bears no linker between the core molecule and the cytotoxic drug may affect its biological activity and that would justify the unexpected difference between the untargeted and unmodified treatment against the targeted iminoboronate methodology.

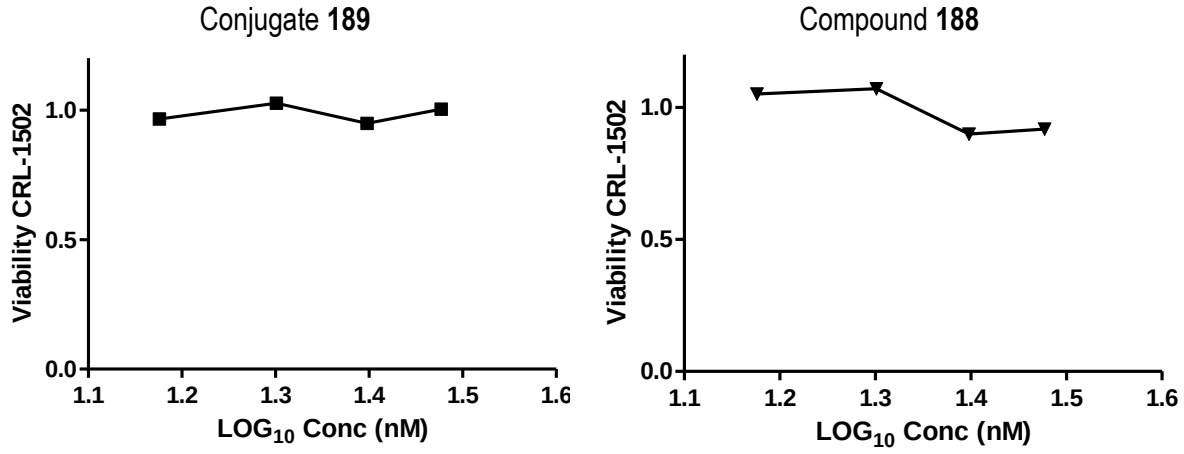


Figure **42** – Viability assay for CRL-1502 (Human Skin Normal Fibroblast) cell model - 24 hs of incubation

Although the conjugate **189** was less potent than PTX alone, the penalty in activity could be well compensated by the increase in safety. Therefore, in an attempt to grant therapeutic value to this approach, the possibility of enhancing the selectivity of internalization towards cancerous cells was evaluated through a viability study with a non-cancerous cell line, determining if the cells were more viable with PTX alone or with the conjugate **189**.

Unfortunately, the viability assay for PTX alone couldn't be done in due time. However, by comparing the conjugate **189** with the ABBA PTX derivative (**188**), the non-cancerous cell line appears to have a higher tolerability for the iminoboronate conjugate than for the unconjugated compound. Having in consideration a report that studied the viability of another human skin fibroblasts cell line (1043SK - proliferating human skin fibroblasts) against PTX, which detected a concentration between 10 and 50 nM to significantly decrease the viability of these cells,[283] we envision that between the evaluated range (10-30 nM), much like the ABBA PTX derivative alone (**188**), isolated PTX will also be more cytotoxic than the conjugate **189**.

IV.4. Conclusions

In this chapter, the synthesis of several compounds with different chemical properties was presented, as well as the resulting biological applications.

Apart from different protein conjugations, interesting applications were obtained. Namely, an artificial glycosylation of lysozyme, three new fluorescent probes for biomolecule conjugation, PEGylation of several biomolecules and a cytotoxic conjugate with a possible therapeutic application.

The PEGylation of insulin with its reversibility dependence upon fructose concentration is an interesting method for a pharmaceutical application and the biological studies using a vitamin derivative (folic acid) as partner of conjugation are particularly interesting, as well.

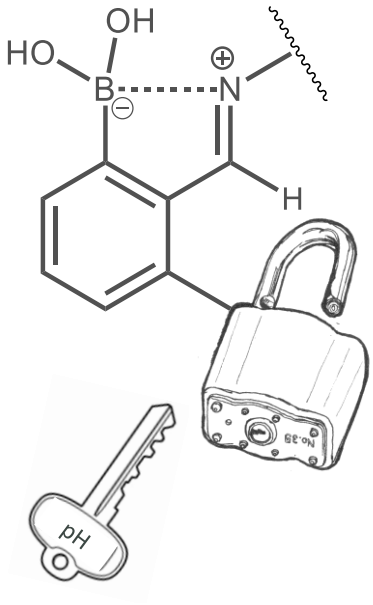
The cell internalization study resulted in an important conclusion, which was that iminoboronates are stable enough to go through cellular internalization. Furthermore, the toxicity assessment of paclitaxel versus paclitaxel conjugated to a folate derivative towards cangerigenous and non-cancerous cell lines was important to assess the possibility of having a greater level of selectivity.

Currently, a study of intracellular reversibility of the conjugate is in progress through the development of an "ON-OFF" probe that is specific for this type of conjugation.

Although, regardless of its outcome, during the process of finding biological applications for iminoboronate methodology, excess of reversibility under certain conditions was observed as the major issue of this bioconjugation system. Therefore, in **Chapter V.** a novel idea currently under study will be discussed.

Chapter V

Searching for pH Switchable Irreversibility



ABSTRACT

Based on the applications developed for 2-carbonylbenzeneboronic acids, it was clear that the applications were limited due to an excessive reversibility that also hampered the ability to separate the conjugated species from the starting materials.

Hence, in this chapter, an ongoing study is presented, where an optimization to improve this bioconjugation system is envisioned and the synthesis of a compound with a possible pH switchable irreversibility is disclosed.

So far the conjugation attempts did not proved to be a successful system, though molecule optimizations are being performed in order to achieve a pH controlled conjugation with iminoboronates as its foundation.

Chapter V.
Searching for pH Switchable Irreversibility

V.1. Introduction

Considering the last set of applications mentioned in **Chapter IV.** (therapeutic applications of bioconjugates), the stability of such molecules is a crucial factor in the design of the constructs, since it significantly affects their potency, selectivity and pharmacokinetics.[123] For instance, antibody-drug conjugates, which are therapeutic bioconjugates with given proofs of effectiveness, require stable linkers and conjugation systems to endure systemic circulation. However, upon cellular internalization and a given activation pathway, those ADCs must be able to release their cytotoxic cargos.[284] Since the stability of conjugation systems was already discussed in **Chapter I.** the different strategies to design such linkers will be briefly commented, since one of these approaches inspired us to develop pH switchable irreversible derivatives of 2-carbonylbenzeneboronic acids.

Hence, three different types of ADC linkers with different cleavage systems can be mentioned. Which are acid-labile linkers, that hydrolyse at mildly acidic environments exploiting the differences in pH between the bloodstream (pH 7.3 – 7.5) and intracellular media (endosomes pH 5.0 - 6.5; lysosomes pH 4.5-5.0); enzyme-labile linkers, that have recognizable peptidic sequences for enzyme degradation mediated by lysosomal proteases; and disulfide-containing linkers, that are cleaved in the presence of an intracellular thiol, like gluthathione.[123,285]

Of all the mentioned linker strategies, acid-labile hydrazone linkers were the first to be used in the construction of ADCs,[286] and until today, still represent one of the most applied strategies for cleavable linkers. However, these linkers have shown non-specific release of the drug in clinical studies, have lower specificity *in vitro* and higher levels of toxicity *in vivo* when compared to highly stable linkers.[287] One of those stabler linkers is based on a fairly recent concept of developing noncleavable (irreversible) linkers designed not to influence the cytotoxicity of the ADC.[286]

Nevertheless, the utility of reversible versus irreversible linkers has been a topic of discussion during the last years, since ADC linkers are considered to be the crucial structure that limits higher efficacy and tolerability levels. Therefore, different strategies to design novel bioconjugates have to be developed in order to cover the gaps in the present state of the art.

V.2. Goals

Considering the facts mentioned in the introduction and on the results from biological applications presented in **Chapter IV.** the main objective outlined for this chapter was to improve the iminoboronate bioconjugation.

This goal was focused in a way as to avoid extra linkers and for the conjugation system itself to serve as a cleavable moiety triggered by an intercellular activation pathway (mildly acidic pH from endosomes and lysosomes).

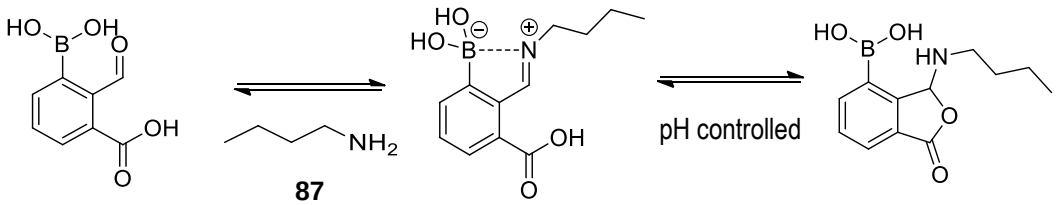
The preliminary results presented herein are a part of an ongoing project that requires improvement and lead us to the future perspectives of this study.

V.3. pH Controlled Irreversibility

Driven by the biological limitations that the iminoboronate conjugates presented, taking into consideration the difficulties to separate the conjugates from the unreacted compound and the inability of using the boronic acid in subsequent reactions (due to its importance in stabilizing the imine), other strategies to turn this reversible strategy into an irreversible one were envisioned.

V.3.1. Concept to be developed

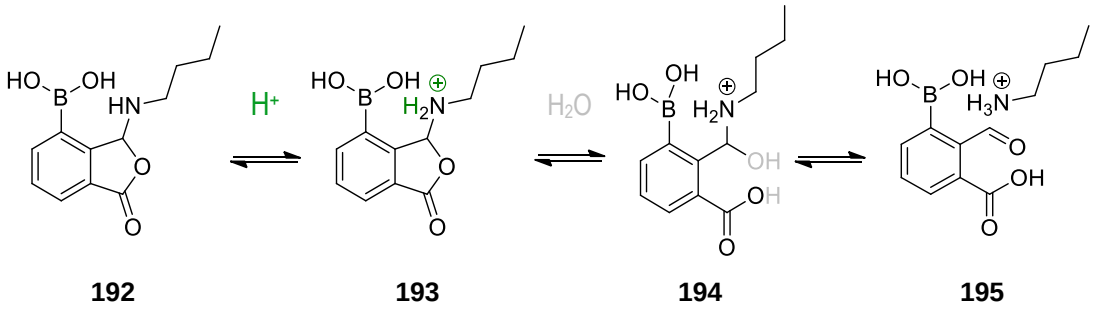
To turn the iminoboronate methodology into an irreversible conjugation method, apart from ideas that were based on reducing strategies (either by adding a second reagent or by synthesizing a compound bearing reducing moieties), the one selected for further development was based on an intramolecular cyclization that could lead to a pH controlled reversibility. Thus, a 3-carboxy-2-formylbenzeneboronic acid was proposed as a 2-FBBA derivative presenting a functional group that could shift between an open and a cyclic form depending upon its protonation state (Scheme **67**).



190 **191** **192**

Scheme **67** – Target molecule (**190**) to obtain a pH controlled cyclization

As depicted in Scheme **67**, upon reaction with a model primary amine, compound **190** is expected to react through the same mechanism as for 2-FBBA to generate the iminoboronate **191**, at near neutral pH. The major difference of this methodology is the anticipated cyclization through acid or base-promoted catalysis that would bring extra stability to transport a given conjugate. However, once formed, acid catalysis can also promote the cleavage of the conjugate as depicted in Scheme **68**. Therefore, this strategy is based on the introduction of a carboxylic acid that will act as a pH "lock" for iminoboronates.

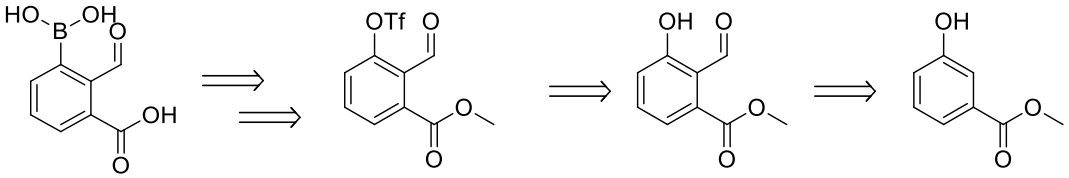


192 **193** **194** **195**

Scheme **68** – Predicted Mechanism of the conjugate's degradation under acidic pH

V.3.2. Synthetic Pathway towards an Irreversible Molecule

Hence, a few retrosynthetic pathways leading to compound **190** were outlined. Scheme **69** represents the strategy that afforded the best results. In such proposal, regardless of the deprotecting steps required after installing all functionalities into the core molecule (acid, aldehyde and boronic acid), the crucial steps would be: a Miyaura borylation, a triflation and a Duff reaction or hexamine aromatic formylation (from **198** to **197**).

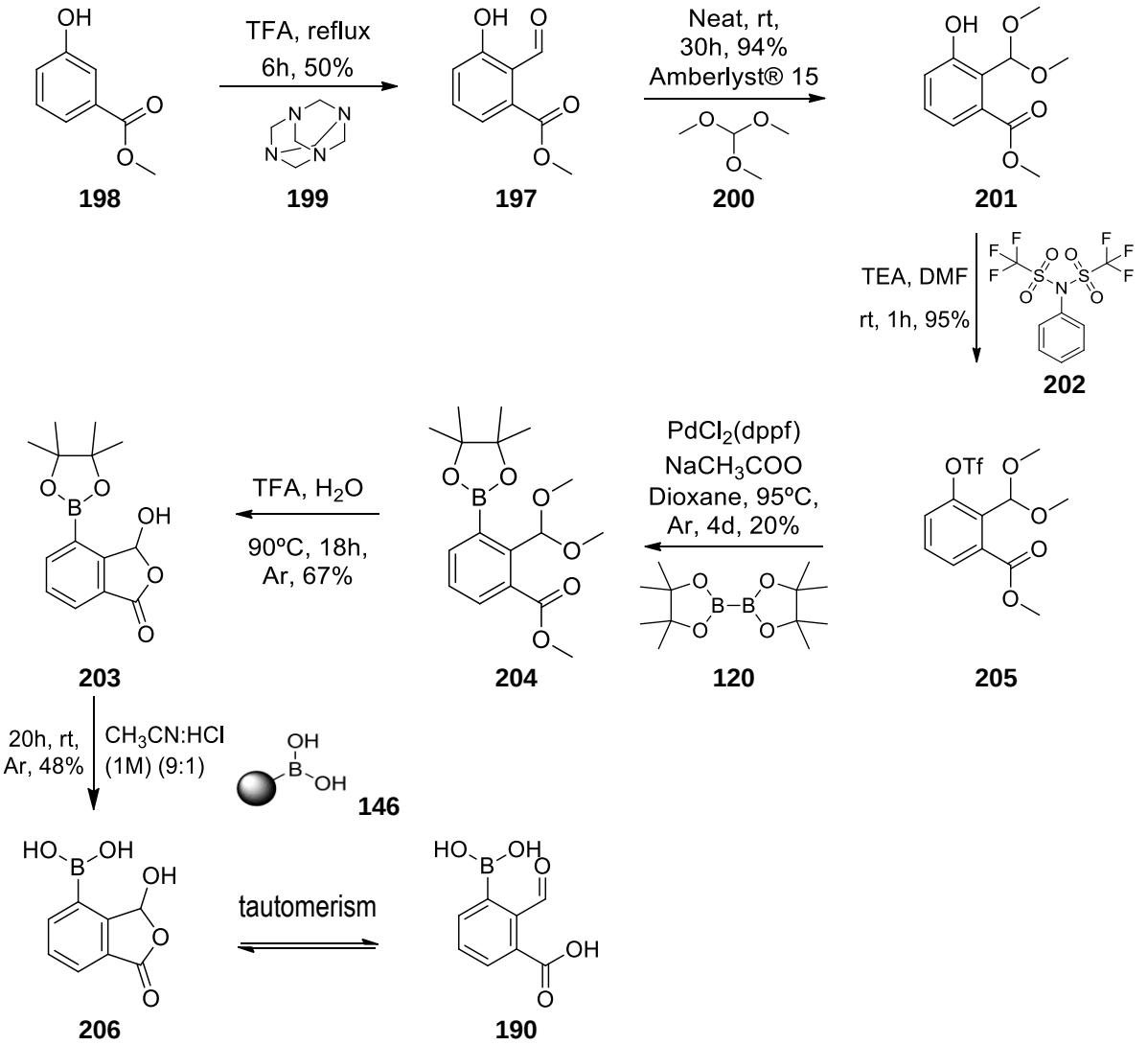


190 **196** **197** **198**

Scheme **69** – Retrosynthetic pathway to synthesize the intended molecule

Based on Scheme **69**, the first chemical reaction (hexamine aromatic formylation) smoothly afforded three different isomers of formylated **198**, in which the major isolated product was **197**. The following step (triflation) did not afford the expected product **196** as the major product and, thus, a carbonyl protecting

step was introduced to avoid any cross-reactions. For that, an acid-catalysed protection with trimethyl orthoformate gave compound **201** in 94% yield. In contrast with the unprotected species, this protected aldehyde, under the same conditions afforded the triflate product in excellent yields. Once again, the Miyaura borylation was the rate-limiting step in the synthesis of *ortho*-carbonylbenzeneboronic acids affording the boronate ester **204** in only 20% yield. However, at this stage, the molecule already had all the organic functionalities required for the envisioned strategy and all the remaining steps would only be deproctection steps (from two esters to acids and an acetal to an aldehyde).



Scheme **70** – Synthetic route to target molecule (**190**) to obtain a pH controllable cyclization

Since acid-catalysed hydrolysis is known to deprotect all the functionalities present in **204**, a reported procedure of ester hydrolysis was followed, in which a major product (**203)** precipitated from the reaction mixture. Longer reaction times were attempted in an effort to also hydrolyze the boronate ester and, thus,

generate **206** (a tautomer from the intended molecule) in a single step, but this was not possible and a subsequent deprotection step was required to complete this synthesis. Compound **190** was not isolated, though its presence in solution was verified by reacting compound **206** with primary amines to test its ability of generating imines or the cyclized *y*-lactone depicted in Scheme **67**.

V.3.3. Preliminary Results

Starting from the evaluation of **206** as being able to generate the aldehyde in solution, the most simple reaction of imine formation was performed. For that, this compound was dissolved in aqueous media (D_2O) and a primary amine (1-butylamine) was added to this solution and left reacting at room temperature. After 18 h, the reaction mixture was evaluated by performing ^{1}H-NMR, which led to the following conclusions: no aldehyde proton was detected (as in all reactions between 1-butylamine and 2-carbonylbenzeneboronic acid), and the conversion to the cyclized form (**192**) was also unobserved. In this spectrum, the only species present were the reagent (**206**) and the imine in 35% conversion (**191**).

In order to promote the cyclization, a disruption of the interaction between boron and nitrogen was attempted by adding a single equivalent of fructose to the reaction mixture and register another ^{1}H-NMR to report any differences. From this second study, it is concluded that the imine hydrolysed completely, though through this experiment it seems that the cyclized *y*-lactone observed is the one from the starting material instead of the one from the expected product (**192**). Nevertheless, driven by these promising results of imine formation, a study at different pHs was undertaken to evaluate its influence on imine generation and cyclization.

Based on previous assays for 2-carbonylbenzeneboronic acid, four reactions between compound **206** with 1-butylamine were performed under naturing conditions (ammonium acetate buffer with different pHs at 25 ºC). From this study, the cyclized form remained undetected and imine formation was observed to be more favourable with increasing pHs (Figure **43**). As a control of this reaction, 2-carboxybenzaldehyde reacted under the exact same conditions, only to afford no imine or *y*-lactone at any of the studied pHs.

From this study, it was concluded that possibly there is too much ring restrainment to form the *y*-lactone and, therefore, a new set of compounds were envisioned to add an extra atom to the carboxy moiety for generating a six-membered ring, instead of the five-membered ring depicted in Scheme **67**.

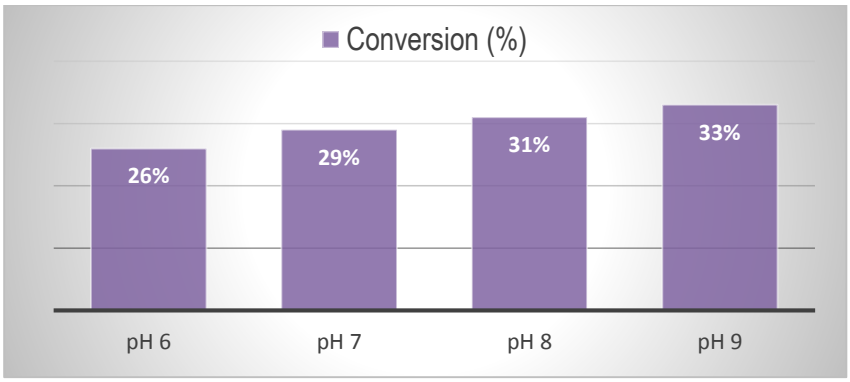


Figure **43** – Conversion of Imine Formation with **206** in percentage

V.3.4. Molecule's Modification to Promote Cyclization

In order to improve the conjugation based on the carboxylic acid derivative of 2-FBBA and promote the cyclization pathway mentioned above, a plan was developed to generate a six-membered ring on the cyclized form instead of the *γ*-lactone. This suggestion was made since the interaction between N-B to enhance imine formation can hamper the cyclization due to an expected thermodynamically disfavoured intermediary with a cluster-like conformation, as depicted in Figure **44**.

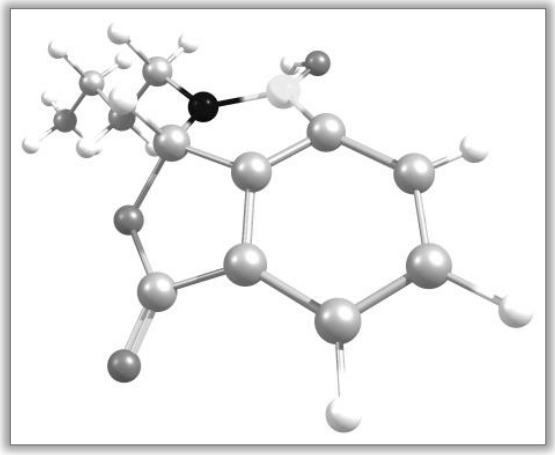
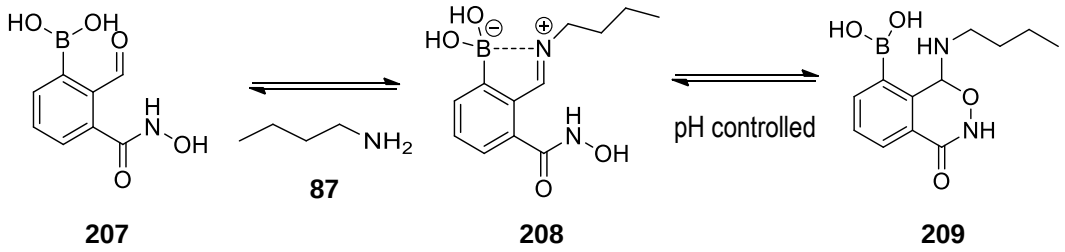


Figure **44** – Optimized geometry (PBE0/6-31G**) of the expected intermediary between the imine (**191**) and the final product (*γ*-lactone, **192**)

Therefore and based on the fact that compound **206** was already synthesized, a path to take advantage of this compound was envisioned, in which the cyclization pathway could be favoured (without

changing the properties intended for this strategy). This pathway was based on the synthesis of a hydroxamic acid instead of the carboxylic acid as demonstrated by Scheme **71**.



207 **87** **208** **209**

Scheme **71** – Improved intended molecule (**207**) to obtain a pH controlled cyclization

The synthesis and optimization of reaction conditions to obtain compound **207** are under development and no crucial information can yet arise from discussing the results obtained this far.

V.4. Conclusions

In this chapter, an acknowledgement of the limitations of iminoboronate bioconjugation system is presented and as a possible solution, an ongoing work based on a potentially pH controllable reversibility was discussed.

So far, an acidic derivative of 2-formylbenzeneboronic acid was already synthesized, though it did not work as well as envisioned. Currently, modifications of this derivative are being developed to decrease the expected ring tension of the intermediaries required to form the cyclized amine.

Therefore, we began to improve this bioconjugation method in order to have a broader number of biological applications that could be applied for this interesting selective conjugation of primary amines of biomolecules.

General Conclusions

Taking into account the main goal of this project, *i.e.* the development of several new reagents to modify non-recombinant proteins, it can be stated that the goal was successfully achieved.

Manipulation of non-recombinant proteins was completed through a selective and rather controllable modification of lysine residues (and the *N*-terminal motif). Iminoboronate bioconjugation method was considered a valuable tool to modify lysine residues and it is considered to be very selective towards primary amines.

From this proven methodology, different synthetic procedures were investigated so that chemical entities could be incorporated into structures that also presented 2-carbonylbenzeneboronic acids. From these chemical derivatives of formyl/acetyl boronic acids, different biological applications were pursued and interesting results were achieved. Namely, one that proved the level of stabilization of iminoboronate modification through a cell internalization procedure and another that compared a conjugated and unconjugated cytotoxic drug to a vitamin derivative in order to confirm if a greater level of selectivity could be attained.

Finally, recognizing the lack of stability of our bioconjugation method under certain conditions, chemical improvements for this methodology were and are being pursued so that a pH switchable lock could be obtained. This envisioned irreversible linkage in the conjugate could be controlled upon specific pH conditions.

Currently, two projects are moving in parallel, one that will allow the conformation of iminoboronate conjugate release after cell internalization, through the development of an “ON-OFF” probe for this technology and another based on optimizing the 2-carbonylbenzeneboronic acid core to achieve a more controllable conjugation.

Chapter VI

Materials and Methods

Chapter VI.
Materials and Methods

VI.1. General Remarks

All chemicals and solvents were of analytical reagent grade and were purchased from Alfa Aesar or Sigma-Aldrich. Tetrahydrofuran was distilled from sodium-benzophenone system prior to use. Dichloromethane and diethyl ether were distilled over CaH_2 prior to use. Dioxane was distilled over metallic sodium prior to use. All reactions were performed in oven-dried glassware or in sterilized 1.5-2 mL eppendorf tubes and, generally, performed under inert atmosphere.

Thin layer chromatography was performed using Merck silica gel 60F254 aluminium plates and visualized by UV light, iodine, potassium permanganate dip, *p*-anisaldehyde dip, ninhydrin dip and dinitrophenylhydrazine dip. Flash column chromatography was performed using Merck silica gel 60 (230-400 mesh ASTM) eluting with various solvent mixtures and using an air aquarium pump to apply pressure.

NMR spectra were obtained on a Bruker 400 Ultra-Shield (400 MHz), a Bruker Avance 500 (500 MHz) and a Bruker Avance III 600 Cryo (600 MHz) using deuterated chloroform, acetone, methanol or dimethylsulfoxide as solvents and $(CH_3)_4Si$ (^1H) as internal standard; chemical shifts, δ, are expressed in ppm, and coupling constants, *J*, are expressed in Hz. Multiplicities are given as: s (singlet), d (doublet), dd (double doublet), dt (double triplet), t (triplet), td (triple triplet), tt (triple triplet), q (quartet), quint (quintuplet) and m (multiplet).

The infrared spectra were collected on a FTIR Shimadzu IRAffinity-1 infrared spectrophotometer. The spectra were determined using in KBr disks or thin films between NaCl plates. Only the most significant absorption bands are reported.

The UV-visible spectra were recorded on a Shimadzu UV-1603 UV-visible spectrometer with a temperature controlled (25 ºC) cell holder.

Low resolution ESI mass spectra were carried on a linear trap mass analyser (ThermoFinnigan LTQ) equipped with an orthogonal electrospray interface. The linear trap mass spectrometer was operated in the negative mode with the ion source voltage set to 5 kV, capillary voltage -49 V, tube lens offset -250 V, capillary temperature 350 ºC, sheath gas flow rate 20 (arbitrary units), auxiliary gas flow rate 10

(arbitrary units) and sweep gas flow rate 5 (arbitrary units). Mass spectra were acquired over the scan range *m/z* 200 to 1000.

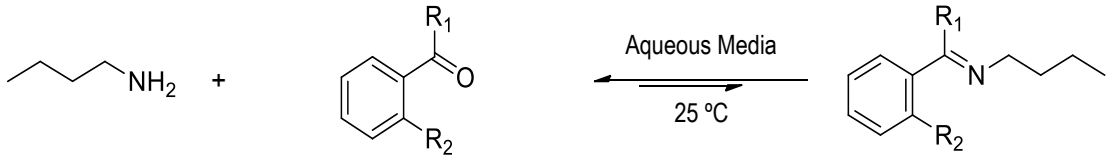
Precursor ions were selected with an isolation width of 2 *m/z* units and activated for 30 ms. Collision-induced dissociation (CID) experiments used helium as the collision gas and normalised collision energy settings were in the range 30-80%, depending on the compound. Data were processed using Xcalibur 1.3 software (ThermoFinnigan, now ThermoScientific).

High resolution mass spectrometry was performed on an equipment with the following specifications: FTICR-MS Bruker Daltonics Apex Qe, Apollo II combi source, 7 Tesla Magnet. Native mass spectrometry was performed, thus maintaining secondary and tertiary structures of the protein during the process of ionization, which was operated in positive mode. Data Analysis was used as the deconvolution software.

VI.2. Experimental Section of Chapter II

VI.2.1. Experimental Details of Experimental Results and Optimization

VI.2.1.1. Reactions with aboronated compounds



87

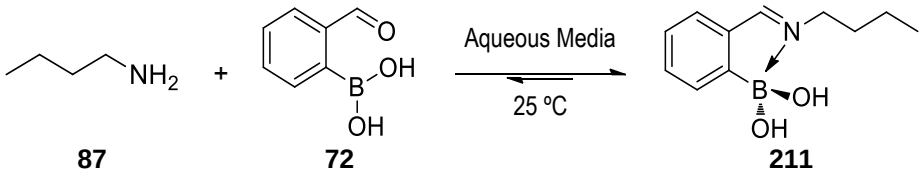
77 (R_1 = -H, R_2 = -H)
78 (R_1 = -H, R_2 = -COOH)
79 (R_1 = -H, R_2 = -OH)
80 (R_1 = -CH_3, R_2 = -H)

210

Scheme **72** – Reactions with aboronated compounds

Formaldehyde (**77**), 2-carboxybenzaldehyde (**78**), salicylaldehyde (**79**) and acetophenone (**80**) (0.33 mmol) were added to a 10 mL round bottom flask and then dissolved in 2 mL of water (0.165 M). Afterwards, 1 eq of 1-butylamine (**87**) was added to the same flask and these compounds reacted for 18 h at 25 ºC. At that pre-determined times, one drop of the reaction mixture was taken from the media and diluted in D_2O in order to collect a ^{1}H-NMR spectrum and evaluate the product's conversion. These conversions were calculated using the following formula: integration of *α*-protons from the imine divided by the sum of *α*-protons from the imine and from the amine.

VI.2.1.2. Preliminary reaction with 2-formylbenzeneboronic acid and optimization results

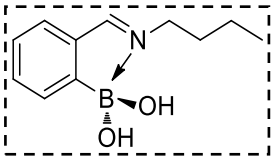


87 **72** **211**

Scheme **73** – Reaction between 2-FBBA and 1-butylamine

2-Formylbenzeneboronic acid (**72**, 50 mg, 0.33 mmol) was added to a 10 mL round bottom flask and then dissolved in 2 mL of solvent (water or phosphate buffer solution [50 mM, pH 8.02]). Afterwards, 1-butylamine (**87**, 33 µL, 0.33 mmol) was added to the same flask and these compounds reacted for 18 h at 25 ºC (Scheme **73**). At that time, one drop of the reaction mixture was taken from the media and diluted in D_2O in order to collect a ^{1}H-NMR spectrum and evaluate the product's conversion based on the comparison of the signal from *α*-protons of 1-butylamine and the same protons from imine, which differ from ~2.5 ppm to ~3.6 ppm, respectively. The conversion in neat water was 49% and in the phosphate buffer 52%.

From an equivalent reaction performed on an organic solvent (dichloromethane), it was possible to isolate the imine by evaporating the solvent and performing a ^{1}H-NMR in $CDCl_3$. Due to a quantitative reaction it was possible to characterize product **211**:

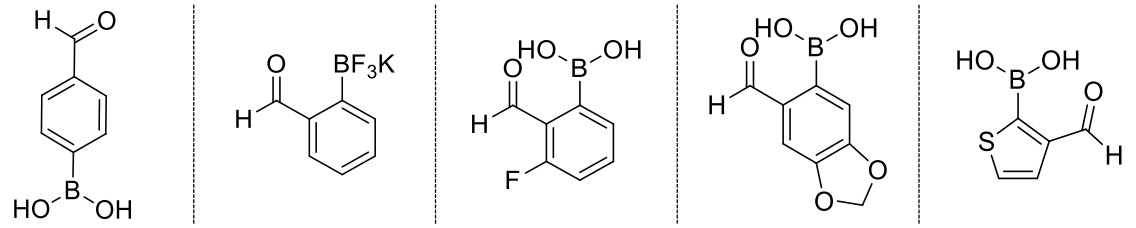


^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.38 (s, 1H, ArC<u>H</u>NCH$_2$-), 7.61 (d, *J*=7.1 Hz, 1H, C<u>H</u>$_{arom}$), 7.49 (d, *J*=7.5 Hz, 1H, C<u>H</u>$_{arom}$), 7.31 (t, *J*=7.3 Hz, 1H, C<u>H</u>$_{arom}$), 7.19 – 7.13 (m, 1H, C<u>H</u>$_{arom}$), **3.59** (t, *J*=7.3 Hz, 2H, =NC<u>H$_2$</u>CH$_2$-), 1.71 – 1.61 (m, 2H, -CH$_2$C<u>H$_2$</u>CH$_2$CH$_3$), 1.27 (dd, *J*=15.1, 7.5 Hz, 2H, -CH$_2$C<u>H$_2$</u>CH$_3$), 0.79 (t, , *J*=7.4 Hz, 3H, -CH$_2$CH$_2$C<u>H$_3$</u>).

^{13}C NMR (100 MHz, $CDCl_3$) δ 165.14 (s, ArC<u>H</u>NCH$_2$-), 138.53 (s, C<u>$_{arom}$</u>), 133.03 (s, C<u>$_{arom}$</u>), 131.58 (s, C<u>$_{arom}$</u>), 130.92 (s, C<u>$_{arom}$</u>), 127.66 (s, C<u>$_{arom}$</u>), 125.73 (s, C<u>$_{arom}$</u>), 52.81 (s, =N<u>C</u>H$_2$CH$_2$-), 31.44 (s, -CH$_2$<u>C</u>H$_2$CH$_2$-), 20.34 (s, -CH$_2$<u>C</u>H$_2$CH$_3$), 13.77 (s, -CH$_2$CH$_2$<u>C</u>H$_3$).

IR (film): 1639.49 cm^{-1} (N=C)

VI.2.1.2.1. *Reactions with formylbenzeneboronic acids*



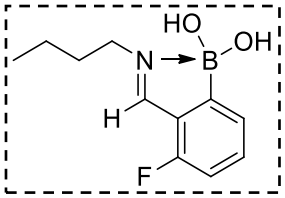
81	**82**	**83**	**84**	**85**
0%	0%	18%	53%	0%

Figure **45** – Compounds **81** to **85** and respective imine conversion from reacting with 1-butylamine in KPi buffer (50.0 mM; pH 8.02), for 18 h at 25 ºC

3-Fluoro-2-formylphenylboronic acid (**83**), 2-formyl-4,5-methylenedioxyphenylboronic acid (**84**), 3-formyl-2-thienylboronic acid (**85**) and 4-formylphenylboronic acid (**81**) were purchased from Sigma-Aldrich and used without further purification. Compound **82** - potassium 2-formylphenyltrifluoroborate - was synthesized according to a procedure described in the literature (Molander, G.A. and Figueroa, R. *Journal of Organic Chemistry* **2006**, *71(16)*, 6135).

A formylboronic acid (0.33 mmol) was added to a 10 mL round bottom flask and then dissolved in 2 mL of KPi buffer (50.0 mM, pH 8.02). Afterwards, 1-butylamine (**87**, 33 µL, 1eq) was added to the same flask and these compounds reacted for 18 h at 25 ºC (Figure **45**). Then, a drop of the reaction mixture was taken from the media and diluted in D_2O in order to collecta ^{1}H-NMR spectrum and evaluate the product's conversion. Results are resumed in Figure **45**.

Characterization of the imine obtained from reaction with compound **83**:

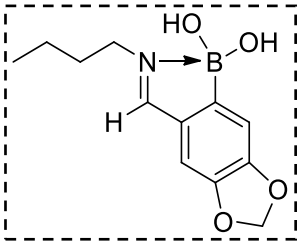


^{1}H NMR (400 MHz, $CDCl_3$) δ 8.47 (s, 1H, ArC<u>H</u>NCH_2-), 7.47 – 7.33 (m, 2H, 2xC<u>H</u>$_{arom}$), 6.96 – 6.81 (m, 1H, C<u>H</u>$_{arom}$), 3.76 (dd, *J* = 17.8, 10.2 Hz, 2H, =NC<u>H</u>$_2$$CH_2$-), 1.85 – 1.73 (m, 2H, -CH_2C<u>H</u>$_2$$CH_2$$CH_3$), 1.43 (dt, *J* = 15.0, 7.4 Hz, 2H, -CH_2C<u>H</u>$_2$$CH_3$), 0.94 (t, *J* = 7.4 Hz, 3H, -$CH_2$$CH_2$C<u>H</u>$_3$).

^{13}C NMR (100 MHz, $CDCl_3$) δ 159.26 (s, Ar<u>C</u>HNCH_2-), 133.75 (s, <u>C</u>$_{arom}$), 133.68 (s, <u>C</u>$_{arom}$), 126.05 (s, <u>C</u>$_{arom}$), 125.13 (s, <u>C</u>$_{arom}$), 125.05 (s, <u>C</u>$_{arom}$), 114.50(s, <u>C</u>$_{arom}$F), 114.30 (s, <u>C</u>$_{arom}$F), 52.87 (s, =N<u>C</u>$H_2$$CH_2$-), 31.41 (s, -$CH_2$<u>C</u>$H_2$$CH_2$-), 20.33 (s, -$CH_2$<u>C</u>$H_2$$CH_3$), 13.81 (s, -$CH_2$$CH_2$<u>C</u>$H_3$).

IR (film): 1641.42 cm^{-1} (CN)

Characterization of the imine obtained from reaction with compound **84**:



^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (s, 1H, ArC<u>H</u>NCH$_2$-), 7.09 (d, *J* = 9.6 Hz, 2H, 2xC<u>H</u>$_{arom}$), 5.94 (s, 2H, -OC<u>H$_2$</u>O-), 3.61 (t, *J* = 7.2 Hz, 2H, =NC<u>H$_2$</u>CH$_2$-), 1.72 (dt, *J* = 15.0, 7.5 Hz, 2H, -CH$_2$C<u>H$_2$</u>CH$_2$CH$_3$), 1.34 (dt, *J* = 14.7, 7.4 Hz, 2H, -CH$_2$C<u>H$_2$</u>CH$_3$), 0.88 (t, *J* = 7.4 Hz, 3H, -CH$_2$CH$_2$C<u>H$_3$</u>).

^{13}C NMR (100 MHz, $CDCl_3$) δ 164.25 (s, ArC<u>H</u>NCH$_2$-), 152.40 (s, C$_{arom}$), 147.40 (s, C$_{arom}$), 132.44 (s, C$_{arom}$), 111.10 (s, C$_{arom}$), 106.04 (s, C$_{arom}$), 101.06 (s, C$_{arom}$), 52.64 (s, =NC$_2$CH$_2$-), 31.52 (s, -CH$_2$C$_2$CH$_2$-), 20.31 (s, -CH$_2$C$_2$CH$_3$), 13.74 (s, -CH$_2$CH$_2$C$_3$).

IR (film): 1641.42 cm^{-1} (CN)

VI.2.1.2.1. *Reagents stoichiometry*

Reactions of 18 h were performed according to the procedure for 2-formylbenzeneboronic acid **(72)**, using KPi buffer (12.5 mM; pH 8.02) and changing equivalents of 2-formylbenzeneboronic acid (**72**) from 1 eq to 20 eq (Scheme **73**). Conversions are resumed in Table **13**.

Table **13** - Reagents stoichiometry from ^{1}H-NMR in D_2O

Quantity of 2-FBBA (72)	Conversions
1 equivalent	58%
5 equivalents	71%
10 equivalents	69%
20 equivalents	68%

VI.2.1.2.2. *Buffer concentration*

Reactions of 18 h were performed according to the same procedure, using 1 equivalent of 2-formylbenzeneboronic acid (**72**), at different concentrations of KPi buffer pH 8.02 (Scheme **73**). Conversions are resumed in Table **14**.

Table **14** - Buffer concentration conversions from ^{1}H-NMR in D_2O

Buffer Concentration	Conversions
0.00 mM KPi	53%
12.5 mM KPi	58%
25.0 mM KPi	50%
37.5 mM KPi	41%
50.0 mM KPi	52%

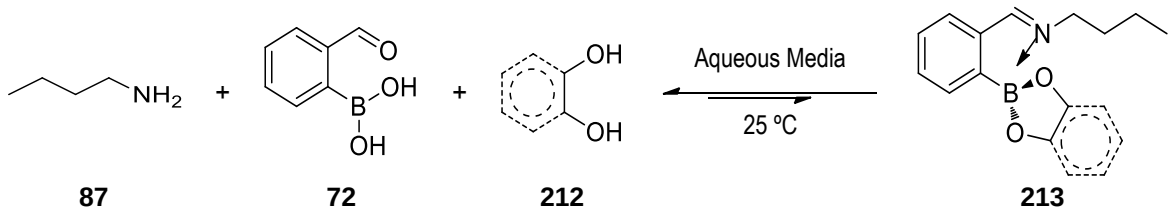
VI.2.1.2.3. *Reaction time*

Reactions were performed according to the general procedure, using 1 eq of 2-formylbenzeneboronic acid (**72**); KPi buffer (12.5 mM; pH 8.02) as solvent and different reaction times (6, 18, 24 and 48 h) (Scheme **73**). Conversions are resumed in Table **15**.

Table **15** - Reaction time conversions from ^{1}H-NMR in D_2O

Reaction Time	Conversions
6 h	56%
18 h	58%
24 h	50%
48 h	53%

VI.2.1.2.4. *Vicinal diols*



87 **72** **212** **213**

Scheme **74** – Reaction between 2-FBBA and 1-butylamine and vicinal diols

2-Formylbenzeneboronic acid (**72**, 25.0 mg, 0.17 mmol) was added to 10 mL round bottom flasks and then dissolved in 2 mL of water. Afterwards, 1 equivalent of cathecol (18.4 mg), *p*-nitrocathecol (26.0 mg) and D-glucose (33.0 mg) were added to these flasks, separately. Then, 1 equivalent of 1-butylamine (**87**, 33 µL) was also added and these compounds reacted for 18 h at 25 ºC. At that time, one drop of reaction mixture was taken from the media and diluted in D_2O to collect a ^{1}H-NMR spectrum and evaluate the product's conversion. No significant imine formation was detected in any of such reactions.

VI.2.1.2.5. *Tautomerism of 2-formylbenzeneboronic acid*

During the scope of the reaction with 2-formylbenzeneboronic acid (**72**), a tautomerism (enhanced by increasing pHs) was detected. This cyclization, between the aldehyde and boronic acid, led to a peak vanishing, related with the formyl proton, and the appearance of a new one at ~6.0 ppm, associated with the proton in the tertiary carbon – compound **86** (Scheme **31**, *p.47*). Also, worth mentioning, a shift of the *α*-protons of 1-butylamine (**87**) was detected whenever the amine was unprotonated or protonated, which differed from ~2.6 to ~2.9 ppm, respectively. This was proven by performing ^{1}H-NMR of reagents 1-butylamine (**87**) and 2-formylbenzeneboronic acid (**72**) at different conditions:

- 1-Butylamine (**87**):

^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.52 (t, *J* = 7.3 Hz, 2H, $NH_2C\underline{H}_2CH_2$-), 1.26-1.17 (m, 4H, $NH_2CH_2C\underline{H}_2C\underline{H}_2$), 0.88 (s, 2H, $N\underline{H}_2CH_2$-), 0.75 (t, *J* = 7.0 Hz, 3H -$CH_2CH_2C\underline{H}_3$).

^{1}H-NMR (400 MHz, D_2O) δ 2.58 (t, *J* = 7.0 Hz, 2H, $NH_2C\underline{H}_2CH_2$-), 1.41-1.20 (m, 4H, $NH_2CH_2C\underline{H}_2C\underline{H}_2$), 0.84 (t, *J* = 7.3 Hz, 3H -$CH_2CH_2C\underline{H}_3$).

At acidic conditions (5% HCl solution)

^{1}H-NMR (400 MHz, D_2O) δ 2.88 (t, *J* = 7.0 Hz, 2H, $NH_2C\underline{H}_2CH_2$-), 1.57-1.51 (m, 2H, $NH_2CH_2C\underline{H}_2CH_2$),1.36-1.30 (m, 2H, $NH_2CH_2CH_2C\underline{H}_2$-), 0.87 (t, *J* = 7.3 Hz, 3H -$CH_2CH_2C\underline{H}_3$).

- 2-Formylbenzeneboronic acid (**72**):

^{1}H-NMR (400 MHz, $CDCl_3$) δ 9.93 (s, 1H, ArC$\underline{H}$O), 8.32 (dd, *J* = 5.6, 3.2 Hz, 1H, C$\underline{H}_{arom}$), 7.99 – 7.88 (m, 1H, C$\underline{H}_{arom}$), 7.76 – 7.70 (m, 2H, 2xC$\underline{H}_{arom}$).

^{1}H-NMR (400 MHz, D_2O) δ 9.90 (s, 1H, ArC$\underline{H}$O), 7.92 (d, *J* = 7.6 Hz, 1H, C$\underline{H}_{arom}$), 7.68 (dd, *J* = 10.6, 4.1 Hz, 1H, C$\underline{H}_{arom}$), 7.58 (dd, *J* = 10.9, 4.1 Hz, 2H, 2xC$\underline{H}_{arom}$)

At basic conditions (5% NaOH solution):

^{1}H-NMR (400 MHz, D_2O) δ 7.43 (d, *J* = 5.7 Hz, 1H, C$\underline{H}_{arom}$), 7.27 (d, *J* = 8.1 Hz, 3H, 3xC$\underline{H}_{arom}$), 6.04 (s, 1H, -BOC$\underline{H}$(OH)-).

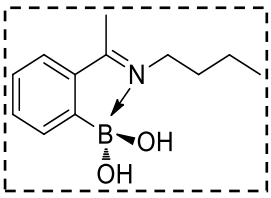
VI.2.1.1. Preliminary reaction with 2-acetylbenzeneboronic acid and optimization results

2-Acetylbenzeneboronic acid (**88**, 55.0 mg, 0.33 mmol) was added to a 10 mL round bottom flask and then dissolved in 2 mL of water or KPi buffer pH 8.02 (50.0 mM). Afterwards, 1 equivalent of 1-butylamine (**87**, 33 µL) was added to the same flask and these compounds reacted for 18 h at 25 ºC (Scheme **32**, *p.47*). At that time, one drop of each reaction mixture was taken from the media and diluted in D_2O in order to collect a ^{1}H-NMR spectrum and evaluate the product's conversion based on the comparison of the signal from *α*-protons of 1-butylamine or the same protons from imine (**89**), which differ from ~2.5 ppm to ~3.6 ppm, respectively. Conversions are resumed in Table **16**.

Table **16** - First Reactions conversions from ^{1}H-NMR in D_2O

Solvent	Conversions
Water	88%
KPi buffer pH 8.02 (50.0 mM)	88%

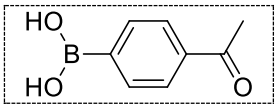
- Characterization of compound **89**:



^{1}H NMR (400 MHz, D_2O) δ 7.61 (d, *J* = 7.6 Hz, 1H, C<u>H</u>*arom*), 7.50 – 7.42 (m, 2H, 2xC<u>H</u>*arom*), 7.35 – 7.28 (m, 1H, C<u>H</u>*arom*), 3.60 – 3.49 (m, 2H, =NC<u>H</u>$_2$CH$_2$-), 2.42 (s, 3H, -CNC<u>H</u>$_3$), 1.69 – 1.57 (m, 2H, -CH$_2$C<u>H</u>$_2$CH$_2$CH$_3$), 1.40 – 1.30 (m, 2H, -CH$_2$C<u>H</u>$_2$CH$_3$), 0.88 (t, *J* = 7.4 Hz, 3H-CH$_2$CH$_2$C<u>H</u>$_3$).

^{13}C NMR (100 MHz, D_2O) δ 176.72 (s, ArC<u>H</u>NCH$_2$-), 139.06 (s, C<u>arom</u>), 132.41 (s, C<u>arom</u>), 128.19 (s, C<u>arom</u>), 127.52 (s, C<u>arom</u>), 123.98 (s, C<u>arom</u>), 44.43 (s, =N<u>C</u>H$_2$CH$_2$-), 30.92 (s, -CH$_2$<u>C</u>H$_2$CH$_2$-), 20.09 (s, -CH$_2$<u>C</u>H$_2$CH$_3$), 19.12 (s, ArCN<u>C</u>H$_3$), 12.98 (s, -CH$_2$CH$_2$<u>C</u>H$_3$).

IR (film): 1647.21 cm^{-1} (CN)



90
0%

Figure **46** - Compound **90** and conversion from reacting it with butylamine in water, for 18 h at 25 ºC

Using the same protocol, 4-acetylbenzeneboronic acid (**90**, 0.33 mmol) was reacted with 1 equivalent of 1-butylamine (**87**) in water, only to find that no imine was detected by ^{1}H-NMR after 18 h of reaction at room temperature (Figure **46**).

VI.2.1.1.1. *Kinetic study*

2-Acetylbenzeneboronic acid (**88**, 11.0 mg, 0.067 mmol) was added to a NMR tube and then dissolved in 0.4 mL of D_2O. Afterwards, at room temperature, 1 equivalent of 1-butylamine (**87**, 7 µL) was added to the same tube and several ^{1}H-NMR were performed to study the reaction's kinetics (Scheme **32**, *p.47*). Results are shown in Table **17**.

Table **17** – Kinetic study conversions from ^{1}H-NMR in D_2O

Time	Conversions
15 minutes	81%
90 minutes	83%
180 minutes	84%
480 minutes	84%
1440 minutes	84%

VI.2.1.1.2. *Influence of pH*

2-Acetylbenzeneboronic acid (**88**, 55.0 mg, 0.33 mmol) was added to a 10 mL round bottom flask and then dissolved in 2 mL of KPi buffer (50.0 mM) at different pH's. Afterwards, 1 equivalent of 1-butylamine (**87**, 33 µL) was added and these compounds reacted for 24 h at 25 ºC (Scheme **32**, *p.47*). At that time, one drop of reaction mixture was taken from the media and diluted in D_2O to collect a ^{1}H-NMR spectrum and evaluate the product's conversion. Also, 1 week later, the same tube was re-submitted to collect more information regarding the stability of the formed imine (**89**). Conversions are resumed in Table **8** (*p.49*).

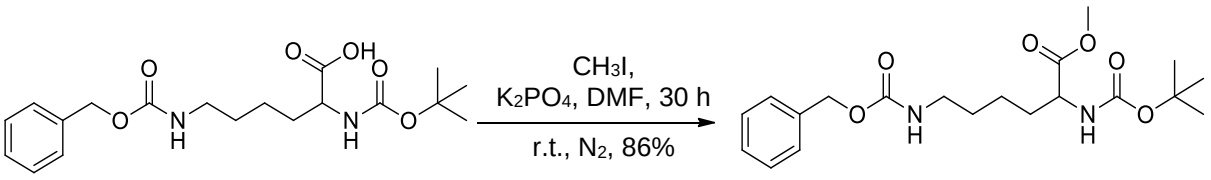
VI.2.1.2. Reactions of 2-carbonylbenzeneboronic acids and biomolecules

VI.2.1.2.1. *Protected lysine*

- Synthesis of Boc-Lys-OCH_3

Boc-β-Lys(Z)-OH (**214**) was purchased from Sigma-Aldrich, and two reactions were made to synthesize Boc-Lys-OCH_3. First, *C*-terminal was protected with iodomethane according to an existing procedure, obtaining compound **215** in 86% yield (Scheme **75**, Liu, R. *et al. Angewandte Chemie* **2010**, 122, 3279), and afterwards performed a hydrogenation catalyzed by Pd/C 20% (m/m) in MeOH, obtaining compound **91** in 81% yield (Scheme **76**, Climie,I.J.; Evans,D.A. *Tetrahedron*; **1982**, *38*, 697). NMR characterization of both compounds in accordance with the ones described in literature.

Step 1 – *C*-terminal protection of Boc-Lys(Z)-OH

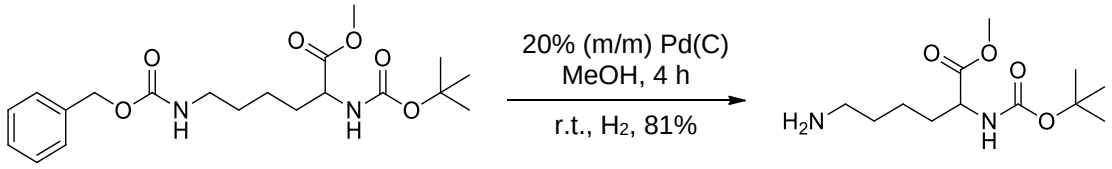


214 **215**

Scheme **75** – *C*-terminal protection of Boc-Lys(Z)-OH

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.21 (m, 5H, C$\underline{H}_{arom}$ Cbz), 5.09 (m, 2H, -CH_2- Cbz), 4.24 (dd, *J* = 13.0, 8.0 Hz, 1H, -$CH_2C\underline{H}$($COOCH_3$)NH-), 3.68 (s, 3H, -OCH_3), 3.14 (dd, *J* = 12.9, 6.5 Hz, 2H, NHC$\underline{H}_2CH_2$), 1.74 (d, *J* = 5.6 Hz, 1H, -$NHCH_2C\underline{H}_{2a}CH_2$-), 1.61 (td, *J* = 14.4, 8.0 Hz, 1H, -$NHCH_2C\underline{H}_{2b}CH_2$), 1.55 – 1.44 (m, 2H, - $CH_2C\underline{H}_2CH_2$-), 1.40 (s, 9H, -CH_3 -^{t}Bu), 1.34 (d, *J* = 6.8 Hz, 2H, -$CH_2C\underline{H}_2CH$-).

Step 2 - Cbz Deprotection of Boc-Lys(Z)-OCH_3



216 **91**

Scheme **76** – Cbz Deprotection of Boc-Lys(Z)-OCH_3

^{1}H NMR (400 MHz, D_2O) δ 4.09 (m, *J* = 9.0, 5.1 Hz, 1H, -CH_2C<u>H</u>($COOCH_3$)NH-), 3.72 (s, 3H, -OCH_3), **2.72** (t, *J* = 7.3 Hz, 2H, -NHC<u>H_2</u>CH_2CH_2-), 1.78-1.46 (m, 4H, -$NHCH_2$C<u>H_2</u>C<u>H_2</u>-), 1.45-1.32 (m, 12H, -CH_2C<u>H_2</u>C<u>H</u>- and -^{t}Bu).

- Reaction between Boc-Lys-OCH_3 and 2-carbonylbenzeneboronic acids

Boc-Lys-OCH_3 (40.0 mg, 0.15 mmol) was added to an eppendorf and dissolved in 0.92 mL of D_2O. Afterwards, 1 equivalent of boronic acid was added and left to react for 16 h at 25 ºC (Scheme **33**, *p.49*). At the end, 0.4 mL of the reaction mixture were removed and a ^{1}H-NMR spectrum was collected and the conversions determined. Conversions are based on the comparison of the signal from *α*-protons of Boc-Lys-OCH_3 or the same protons from imine, which differ from ~2.7 ppm to ~3.6 ppm, respectively. Results are resumed in Table **9** (*p.49*).

VI.2.1.2.2. *Somatostatin*

Somatostatin was purchased from Sigma-Aldrich and used without further purification.

- General procedure for reactions with Somatostatin

2-Formylbenzeneboronic and 2-acetylbenzeneboronic acids (50.0 mM) were reacted with 10 μM of somatostatin in NH_4HCO_3 buffer (10.0 mM, pH 7.8) at room temperature (Scheme **34**, *p.50*) After 5 minutes of reaction the solutions were evaluated by performing an ESI-MS and the conjugated species were detected. Low resolution ESI-MS results and performed fragmentations are resumed in Table **18** for 2-formylbenzeneboronic acid (**72,** 2-FBBA) and in Table **19** for 2-acetylbenzeneboronic acids (**88,** 2-ABBA).

Table **18** – Low resolution ESI-MS results of conjugation between SST and 2-FBBA (**72**)

m/z	Intensity	Interpretation	Fragmentations (MS/MS)	Interpretation
1635.72	22%	SST	-	-
1731.83	38%	SST + 2-FBBA – 2xH_2O	-	-
1750.70	65%	SST + 2-FBBA – 1xH_2O	-	-
1845.77	23%	SST + 2x(2-FBBA) – 3xH_2O	-	-
1863.78	100%	SST + 2x(2-FBBA) – 2xH_2O	1827	(- 2(H_2O)
1882.71	50%	SST + 2x(2-FBBA) – 1xH_2O	-	-
1977.77	43%	SST + 3x(2-FBBA) – 3xH_2O	-	-
1996.78	58%	SST + 3x2-FBBA – 2xH_2O	1978	- 1xH_2O
			1960	- 2xH_2O
			1942	- 3xH_2O
			1828	**- 1x2-FBBA -2xH_2O**

Table **19** – Low resolution ESI-MS results of conjugation between SST and 2-ABBA (**88**)

m/z	Intensity	Interpretation	Fragmentations (MS/MS)	Interpretation
1635.67	12%	SST	-	-
1763.78	28 %	SST + 2-ABBA – 1xH_2O	-	-
1781.85	48 %	SST + 2-ABBA	-	-
1910.87	88 %	SST + 2x(2-ABBA) – 1xH_2O	1874.75	- 2xH_2O
			1856.81	- 3xH_2O
1927.84	100 %	SST + 2x(2-ABBA)	-	-

VI.2.1.2.3. *Proteins*

Lysozyme, from chicken egg white; cytochrome c and myoglobin, from equine heart and ribonuclease A, from bovine pancreas, were purchased from Sigma-Aldrich and used without further purification.

- General Procedure for reactions with Lysozyme

2-Formylbenzeneboronic and 2-acetylbenzeneboronic acids (10 and 20 mM, respectively) reacted with 10 μM of lysozyme in $NH_4CH_3CO_2$ buffer (20.0 mM, pH 7.0) at room temperature. After 30 minutes of reaction the solutions were evaluated by performing an ESI-FTICR-MS and the conjugated species were detected, up to 6 modifications. Results of reaction with 2-FBBA and 2-ABBA are shown in Figure **47** and Figure **6** (*p.51*), respectively.

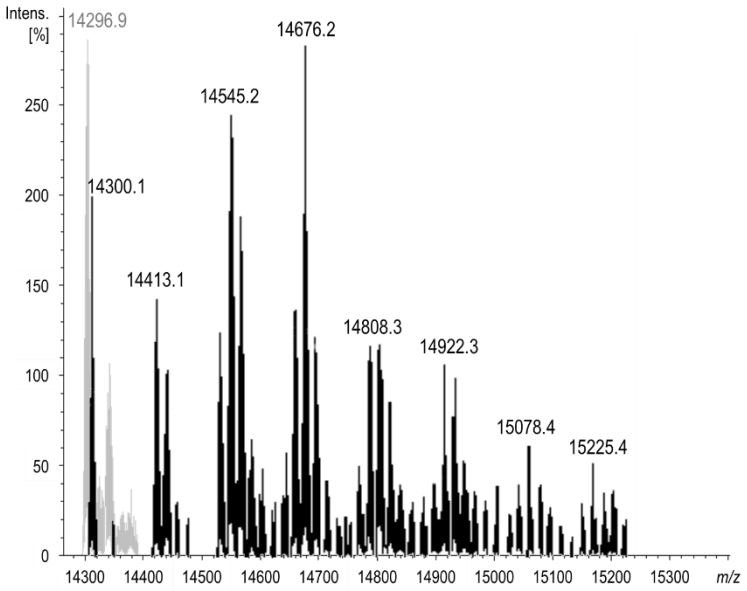


Figure **47** – Overlaid deconvoluted ESI-FTICR-MS spectra. **Grey** - unmodified Lyz; **Black** – Reaction between Lyz (10.0 μM) and 10.0 mM of 2-FBBA (**72**) in ammonium acetate buffer (20.0 mM, pH 7.0)

- Reactions with Different Solvents

2-Formylbenzeneboronic and 2-acetylbenzeneboronic acids (10.0 mM) reacted with 10 μM of lysozyme in $NH_4CH_3CO_2$ buffer (20.0 mM, pH 5.0) or water at room temperature. After 5 minutes of reaction the solutions were evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. Results are shown in Figure **48** and Figure **49**.

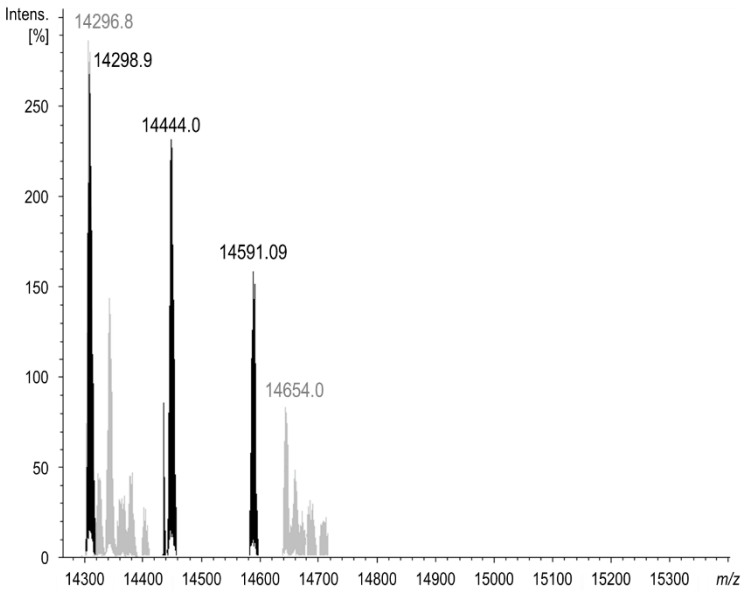


Figure **48** – Overlaid deconvoluted ESI-FTICR-MS spectra. **Grey** - unmodified Lyz; **Black** – Reaction between Lyz (10.0 μM) and 10.0 mM of 2-ABBA (**88**) in ammonium acetate buffer (20.0 mM, pH 5.0)

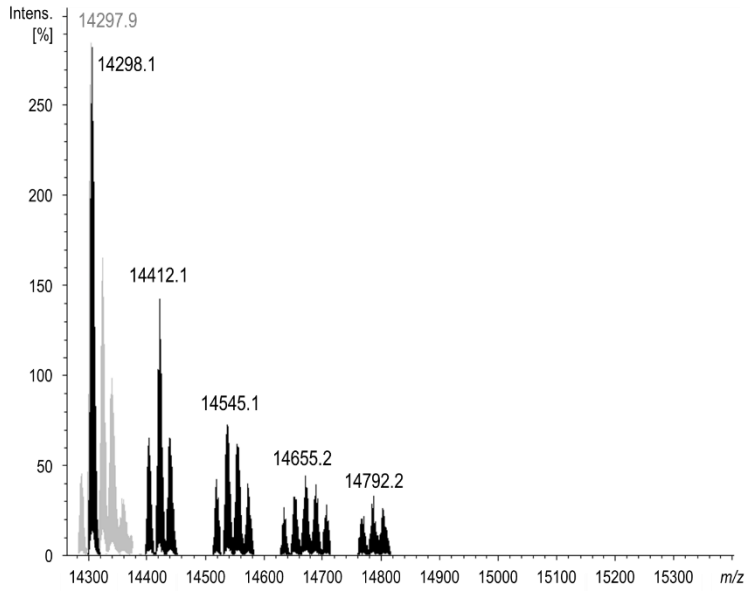


Figure **49** – Overlaid deconvoluted ESI-FTICR-MS spectra. **Grey** - unmodified Lyz; **Black** – Reaction between Lyz (10.0 µM) and 10.0 mM of 2-FBBA (**72**) in neat water

- Reactions with different aldehydes

3-fluoro-2-formylphenylboronic acid (**83**), 2-Formyl-4,5-methylenedioxyphenylboronic acid (**84**), and 3-formyl-2-thienylboronic acid (**85**) (10.0 mM) reacted with 10.0 µM of lysozyme in $NH_4CH_3CO_2$ buffer (20.0 mM, pH 7.0) at 25 ºC. After 5 minutes of reaction the solutions were evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. Results are shown in Figure **7** (*p.52*).

- Reactions with different proteins

2-Formylbenzeneboronic acid (10.0 mM) reacted with 10.0 µM of cytochrome c, ribonuclease A or myoglobin in $NH_4CH_3CO_2$ buffer (20.0 mM, pH 7.0) at 25 ºC. After 5 minutes of reaction the solutions were evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. Results of reaction of 2-FBBA with cytochrome c, ribonuclease A or myoglobin are shown in Figure **8** (*p.53*), Figure **9** (*p.54*) and Figure **10** (*p.54*), respectively.

- Stability of Conjugates

2-Formylbenzeneboronic and 2-acetylbenzeneboronic acids (10.0 mM) reacted with 10.0 µM of lysozyme in $NH_4CH_3CO_2$ buffer (20.0 mM, pH 7.0) at 25 ºC. After determined times of reaction (0, 1, 2, 3 and 4 h) an aliquot was removed from the media and evaluated by performing an ESI-FTICR-MS. Results of reaction with 2-ABBA and 2-FBBA are shown in Figure **50** and Figure **51**, respectively.

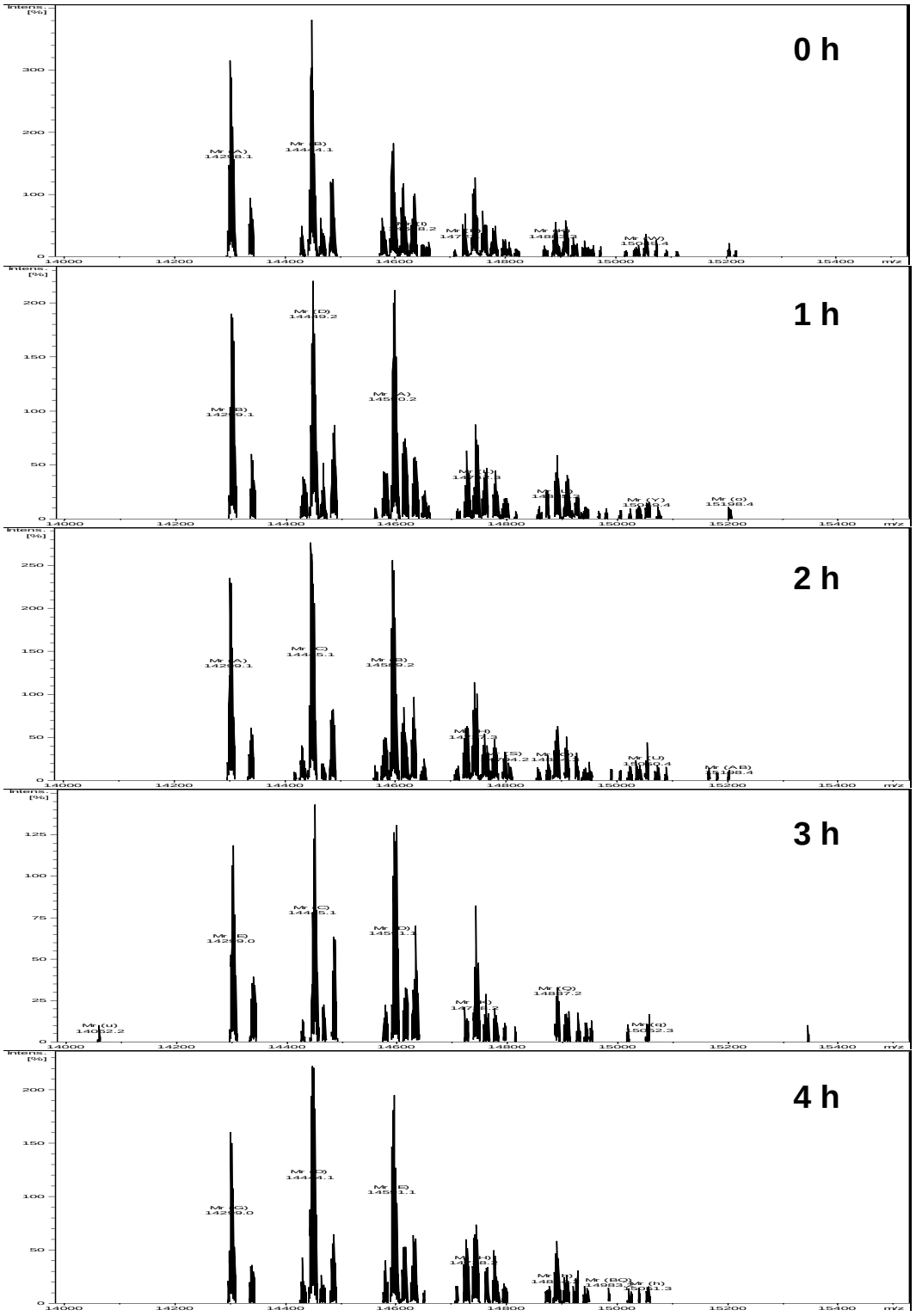


Figure **50** – Deconvoluted ESI-FTICR-MS spectra of reaction between Lyz (10.0 μM) and 10.0 mM of 2-ABBA (**88**) in ammonium acetate buffer (20.0 mM, pH 7.0) after 0, 1, 2, 3 and 4 h

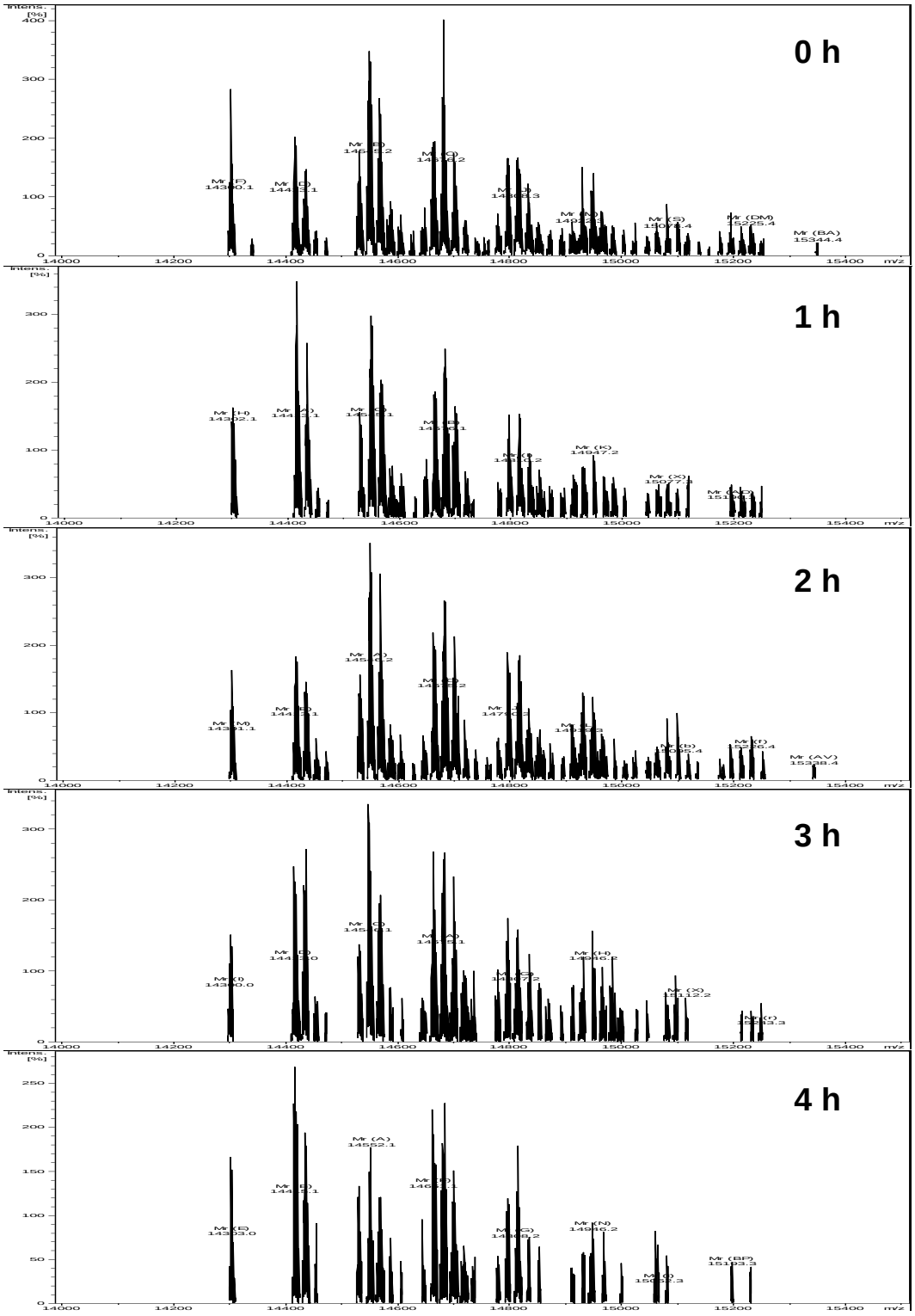


Figure **51** – Deconvoluted ESI-FTICR-MS spectra of reaction between Lyz (10.0 µM) and 10.0 mM of 2-FBBA (**72**) in ammonium acetate buffer (20.0 mM, pH 7.0) after 0, 1, 2, 3 and 4 h

VI.2.2. Experimental Details of Computational Studies

All calculations were performed using the Gaussian 03 software package,[289] and the PBE0 functional, without symmetry constraints. That functional uses a hybrid generalized gradient approximation (GGA), including 25 % mixture of Hartree-Fock[290] exchange with DFT[291] exchange-correlation, given by Perdew, Burke and Ernzerhof functional (PBE).[292,293] The optimized geometries were obtained with a standard 6-31G(d,p) basis set.[294–298] Transition state optimizations were performed with the Synchronous Transit-Guided Quasi-Newton Method (STQN) developed by Schlegel *et al.*,[299,300] after a thorough search of the Potential Energy Surfaces (PES). Frequency calculations were performed to confirm the nature of the stationary points, yielding one imaginary frequency for the transition states and none for the minima. Each transition state was further confirmed by following its vibrational mode downhill on both sides, and obtaining the minima presented on the energy profiles. A Natural Population Analysis (NPA)[301–308] and the resulting Wiberg indices[309–317] were used to study the electronic structure and bonding of the optimized species.

The influence of basis set size and solvent on the calculated reaction mechanism was tested by means of single point energy calculations using a 6-311++G(d,p) basis set and the geometries optimized at the PBE0/6-31G(d,p) level. Solvent effects (water) were considered using the Polarizable Continuum Model (PCM) initially devised by Tomasi and coworkers as implemented on Gaussian 03. The molecular cavity was based on the united atom topological model applied on UAHF radii, optimized for the HF/6-31G(d) level. The free energy values calculated with the better basis set and including solvent effects are similar to the electronic energy values obtained with the smaller basis set in the gas phase, and, hence, these latter are the ones presented in the energy profile. For example, the activation free energy for the formation of **89** from **88** and 1-butylamine is 9 kcal/mol at the PBE0/6-311++G(d,p) (PCM)//PBE0/6-31G(d,p) level, comparing to 12 kcal/mol, the energy barrier calculated at the PBE0/6-31G(d,p) level (cf. Figure **24**). Similarly, the overall energy balance for the reaction is –7 and –10 kcal/mol, by the same order. The same happens when there is no B-atom involved in the reaction, i.e., the reagent is acetophenone (Figure **13**), with a maximum difference of 2 kcal/mol for the relevant energy values calculated at the two levels of theory.

Atomic Coordinates for the Optimized Species are given on the following pages.

Acetophenone and 1-butylamine

Z-Isomer

A

6	0.904045	0.234520	1.529644
6	1.061171	0.246452	0.038543
8	0.202838	0.758626	-0.675786
6	2.242005	-0.402257	-0.598998
6	2.408264	-0.257024	-1.981783
6	3.485676	-0.847315	-2.626018
6	4.404506	-1.601410	-1.896764
6	3.168363	-1.160292	0.124982
6	4.241959	-1.761214	-0.523419
1	1.853905	0.229866	2.066191
1	0.315713	-0.659018	1.799934
1	0.311895	1.101471	1.829188
1	1.674163	0.328077	-2.526301
1	3.611741	-0.724165	-3.697661
1	5.247245	-2.066174	-2.400640
1	3.046729	-1.298292	1.194382
1	4.952927	-2.354222	0.043994
7	-1.769096	-1.661716	1.547371
6	-1.995374	-2.340487	0.279145
6	-3.616197	-3.015710	-1.577137
6	-3.432417	-2.305277	-0.240394
6	-5.049957	-2.970272	-2.090080
1	-2.062279	-0.687174	1.449249
1	-2.354573	-2.077130	2.266163
1	-1.336051	-1.880031	-0.468465
1	-1.664838	-3.383958	0.372832
1	-3.295128	-4.061525	-1.477831
1	-2.945152	-2.564856	-2.320718
1	-3.741630	-1.254224	-0.331491
1	-4.099528	-2.755623	0.509672
1	-5.151889	-3.484563	-3.050872
1	-5.387207	-1.937025	-2.228629
1	-5.737888	-3.447150	-1.383155
8	-2.194218	1.216501	0.691216
1	-1.384087	1.161236	0.149742
1	-2.896339	1.411119	0.065312

TS_{A-B}

6	0.792943	0.512633	1.736147
6	0.385207	0.066997	0.325506
8	-0.055431	1.076949	-0.436714
6	1.480811	-0.749615	-0.352645
6	1.938844	-0.342581	-1.604154
6	2.945990	-1.052128	-2.252597
6	3.501910	-2.181463	-1.658656
6	2.046635	-1.881626	0.240918
6	3.047892	-2.596799	-0.408810
1	1.636767	1.198353	1.635513
1	1.099591	-0.313456	2.387385
1	-0.038290	1.053251	2.197968
1	1.484992	0.541490	-2.039683
1	3.298134	-0.721604	-3.225961
1	4.287528	-2.735958	-2.164070
1	1.712721	-2.213699	1.222326
1	3.478325	-3.475615	0.063287
7	-0.865622	-0.879314	0.556956
6	-1.334652	-1.562965	-0.659263
6	-3.233553	-2.844707	-1.732708
6	-2.716602	-2.167467	-0.467005
6	-4.617035	-3.455316	-1.552056
1	-1.621997	-0.075040	0.812320
1	-0.709380	-1.536158	1.318850
1	-1.340539	-0.792119	-1.436343
1	-0.604437	-2.324104	-0.952136
1	-2.523875	-3.622673	-2.043330
1	-3.256333	-2.111678	-2.549394
1	-3.415992	-1.380083	-0.155895
1	-2.692390	-2.899955	0.352763
1	-4.965345	-3.933236	-2.472298
1	-5.352953	-2.693225	-1.273851
1	-4.614770	-4.215257	-0.763100
8	-2.096515	1.274031	0.777442
1	-1.107897	1.388812	0.112293
1	-2.865384	1.425086	0.220412

B

6	0.791023	0.464546	1.669688
6	0.348820	-0.036669	0.292790
8	-0.005962	1.039158	-0.528733
6	1.489598	-0.785594	-0.393412
6	2.064858	-0.299203	-1.566674
6	3.113616	-0.983577	-2.176553
6	3.600233	-2.163401	-1.623490
6	1.984268	-1.972256	0.155430
6	3.030135	-2.657807	-0.452654
1	1.648396	1.129603	1.547534
1	1.086653	-0.356600	2.330595
1	-0.025812	1.020496	2.139863
1	1.679227	0.620780	-1.990695
1	3.551877	-0.590720	-3.089922
1	4.418194	-2.696398	-2.099653
1	1.550347	-2.371812	1.070115
1	3.401016	-3.578664	-0.011254
7	-0.855314	-0.877371	0.490789
6	-1.340272	-1.552489	-0.712114
6	-3.256622	-2.885060	-1.710914

6	-2.705507	-2.180926	-0.475351
6	-4.624636	-3.512500	-1.477016
1	-2.029362	0.482687	0.944383
1	-0.678211	-1.558158	1.224330
1	-1.403007	-0.790492	-1.496448
1	-0.632803	-2.315996	-1.071130
1	-2.546852	-3.657897	-2.033984
1	-3.317069	-2.166425	-2.538945
1	-3.409841	-1.404986	-0.145007
1	-2.640497	-2.901669	0.353115
1	-4.996416	-4.010827	-2.377330
1	-5.362794	-2.756781	-1.186738
1	-4.586050	-4.258937	-0.676059
8	-2.303889	1.431711	0.962333
1	-0.759201	1.479464	-0.085206
1	-3.078850	1.484532	0.395734
C			
6	0.776923	0.485624	1.612394
6	0.344658	-0.023207	0.236080
8	-0.001563	1.134788	-0.572737
6	1.471274	-0.810356	-0.429858
6	2.102096	-0.366214	-1.590210
6	3.154521	-1.090551	-2.147736
6	3.582671	-2.271869	-1.553550
6	1.897239	-2.008017	0.152868
6	2.944680	-2.732404	-0.402865
7	-0.812898	-0.844953	0.396081
6	-1.416788	-1.365687	-0.825273
6	-3.287712	-2.829269	-1.721365
6	-2.630886	-2.218443	-0.488562
6	-4.517350	-3.664108	-1.387373
1	1.656850	1.132044	1.530090
1	1.027158	-0.346639	2.272708
1	-0.046978	1.054230	2.054443
1	0.741399	1.748860	-0.545801
1	1.748259	0.535262	-2.079863
1	3.633033	-0.731742	-3.054714
1	4.401994	-2.837515	-1.987771
1	1.379223	-2.376549	1.033553
1	3.262148	-3.662618	0.060037
1	-1.508563	-0.314177	0.918396
1	-1.711003	-0.565284	-1.523033
1	-0.668915	-1.977432	-1.344019
1	-2.554326	-3.450876	-2.252398
1	-3.566648	-2.027898	-2.418557
1	-3.367008	-1.601048	0.045950
1	-2.325868	-3.010203	0.207869
1	-4.970732	-4.091175	-2.287344
1	-5.280273	-3.058880	-0.885497
1	-4.262813	-4.493030	-0.717763
8	-2.366162	1.711700	0.860865
1	-1.617699	1.728839	0.239748
1	-2.249299	2.488630	1.413547
TS_{C-D}			
6	0.723270	0.392256	1.748695
6	0.362313	-0.324493	0.481510
8	-0.003502	1.521980	-0.587057
6	1.478572	-0.932090	-0.304076
6	1.863592	-0.449659	-1.556034
6	2.909226	-1.062026	-2.240901
6	3.572590	-2.154441	-1.689044
6	2.146606	-2.029123	0.250801
6	3.187867	-2.638535	-0.441815
7	-0.858754	-0.787173	0.386572
6	-1.318249	-1.545360	-0.769076
6	-3.255483	-2.887754	-1.694681
6	-2.699886	-2.121423	-0.499826
6	-4.637527	-3.471439	-1.431590
1	1.586916	1.040607	1.598069
1	0.985110	-0.348935	2.513826
1	-0.130331	0.971776	2.103443
1	0.544130	2.252316	-0.282191
1	1.330096	0.407863	-1.952280
1	3.206747	-0.680583	-3.213573
1	4.387885	-2.627855	-2.228500
1	1.845597	-2.413176	1.222086
1	3.698835	-3.491594	-0.004895
1	-1.583313	-0.000763	0.708143
1	-1.354300	-0.879328	-1.642072
1	-0.599917	-2.341141	-0.995445
1	-2.561256	-3.694362	-1.967059
1	-3.298762	-2.218519	-2.564090
1	-3.376127	-1.297028	-0.238939
1	-2.651958	-2.780133	0.377526
1	-5.017811	-4.011148	-2.304225
1	-5.357978	-2.683529	-1.186987
1	-4.617219	-4.172215	-0.589728
8	-2.126815	1.327661	0.509910
1	-1.063570	1.589731	-0.062007
1	-2.277951	1.879472	1.281852
D			
6	0.607898	0.347122	1.722736
6	0.162058	-0.472329	0.548302
8	0.620598	2.687734	-0.452461
6	1.256492	-1.148982	-0.213624
6	2.142607	-0.361344	-0.956322
6	3.182395	-0.962417	-1.658746

6	3.354022	-2.344072	-1.616192
6	1.429587	-2.534845	-0.174665
6	2.476980	-3.129038	-0.872789
7	-1.080921	-0.510268	0.254219
6	-1.569964	-1.248369	-0.894548
6	-3.181360	-3.070452	-1.629442
6	-2.527085	-2.352221	-0.454346
6	-4.129681	-4.179109	-1.191264
1	1.204029	1.194540	1.366438
1	1.238337	-0.250653	2.388889
1	-0.253853	0.723999	2.276682
1	0.559820	3.090271	-1.322228
1	1.989517	0.716007	-0.982019
1	3.863871	-0.348218	-2.240634
1	4.170502	-2.808823	-2.161399
1	0.746170	-3.146420	0.408845
1	2.607689	-4.206759	-0.834987
1	-1.843125	1.118922	0.444247
1	-2.120916	-0.533489	-1.520980
1	-0.763592	-1.670240	-1.509183
1	-2.402157	-3.488299	-2.280662
1	-3.727415	-2.339736	-2.240885
1	-3.296297	-1.916015	0.195562
1	-1.978444	-3.076250	0.163644
1	-4.588496	-4.677981	-2.050343
1	-4.937233	-3.783365	-0.565756
1	-3.603771	-4.941343	-0.605913
8	-1.990401	2.086178	0.316219
1	-0.311482	2.535817	-0.193142
1	-2.189736	2.430286	1.191089

***E*-Isomer**

C’

6	1.864655	-0.248751	1.210404
6	0.882296	0.040790	0.075469
8	0.262675	1.330711	0.325362
6	1.654328	0.228100	-1.229684
6	1.946151	-0.874066	-2.035841
6	2.693986	-0.720034	-3.199641
6	3.159619	0.537629	-3.572906
6	2.130089	1.487367	-1.605601
6	2.874442	1.640144	-2.772110
7	-0.067250	-1.021273	-0.092206
6	-0.761774	-1.583420	1.051649
6	-2.573311	-1.437198	2.830954
6	-1.693488	-0.663999	1.853801
6	-3.483375	-0.540930	3.660692
1	2.615396	0.543025	1.246703
1	2.368098	-1.204301	1.044392
1	1.359503	-0.287052	2.182018
1	-0.166378	1.277409	1.186165
1	1.565416	-1.846698	-1.741192
1	2.909857	-1.586534	-3.818547
1	3.740563	0.658394	-4.482838
1	1.904781	2.345670	-0.981652
1	3.232271	2.626066	-3.055750
1	-0.725697	-0.741070	-0.818822
1	-1.363048	-2.415333	0.663862
1	-0.030733	-2.042741	1.728537
1	-1.936703	-2.035122	3.497245
1	-3.180888	-2.157481	2.267211
1	-2.322314	-0.092076	1.158344
1	-1.107125	0.069174	2.431082
1	-4.109842	-1.125803	4.341000
1	-4.148184	0.048005	3.019705
1	-2.902606	0.162271	4.267942
8	-1.537042	0.957323	-1.830070
1	-0.941687	1.005416	-2.584592
1	-1.016985	1.354001	-1.110630

$TS_{C'-D'}$

6	1.821599	-0.080853	1.290200
6	1.012982	-0.205619	0.032111
8	0.088134	1.663712	0.087151
6	1.718063	0.104823	-1.249427
6	1.756220	-0.869805	-2.250848
6	2.462030	-0.644905	-3.429723
6	3.142054	0.553559	-3.616352
6	2.404972	1.308417	-1.444853
6	3.108979	1.529401	-2.620958
7	-0.011950	-1.031382	-0.068635
6	-0.664491	-1.617572	1.088109
6	-2.513152	-1.435887	2.816525
6	-1.685187	-0.701684	1.768063
6	-3.520094	-0.532540	3.516899
1	2.456784	0.802871	1.241261
1	2.471242	-0.959236	1.385403
1	1.194112	-0.018030	2.180831
1	-0.350115	1.751263	0.941050
1	1.236277	-1.808480	-2.088099
1	2.484460	-1.411680	-4.198754
1	3.695138	0.730225	-4.534493
1	2.325783	2.084417	-0.691874
1	3.626409	2.473024	-2.768176
1	-0.742857	-0.563066	-0.837432
1	-1.184443	-2.515355	0.732534
1	0.082820	-1.963041	1.813199
1	-1.844849	-1.889503	3.561533
1	-3.041265	-2.270352	2.336120
1	-2.337080	-0.272137	0.997444

1	-1.166787	0.142212	2.244299
1	-4.107743	-1.084773	4.256578
1	-4.218333	-0.090203	2.798457
1	-3.019293	0.290467	4.038628
8	-1.483903	0.491406	-1.301208
1	-1.343587	0.653854	-2.238993
1	-0.733232	1.230063	-0.640115

D’

6	1.998909	-0.252335	1.373583
6	1.269567	-0.660371	0.123479
8	-0.158767	2.114325	0.509441
6	1.827357	-0.178055	-1.170899
6	1.648612	-0.940408	-2.332223
6	2.141037	-0.494911	-3.553278
6	2.813416	0.723626	-3.632722
6	2.509383	1.040471	-1.259009
6	2.991069	1.490195	-2.484378
7	0.179119	-1.329727	0.056891
6	-0.496012	-1.818437	1.239420
6	-2.402271	-1.366158	2.859755
6	-1.660342	-0.898606	1.612761
6	-3.572373	-0.460278	3.221582
1	3.074349	-0.181038	1.198187
1	1.823791	-0.938420	2.203270
1	1.623095	0.736525	1.663543
1	-0.861641	2.649180	0.886970
1	1.122684	-1.887042	-2.253878
1	2.006139	-1.101758	-4.444207
1	3.197767	1.073195	-4.586675
1	2.620789	1.661197	-0.376140
1	3.502116	2.446812	-2.542441
1	-0.912616	-0.301924	-1.119598
1	-0.887872	-2.816459	1.004817
1	0.168238	-1.932407	2.107946
1	-1.702147	-1.416258	3.704672
1	-2.765487	-2.391435	2.706082
1	-2.355770	-0.843857	0.765243
1	-1.271344	0.118206	1.751056
1	-4.091049	-0.812294	4.118624
1	-4.304138	-0.417587	2.407769
1	-3.233209	0.563592	3.413147
8	-1.312712	0.513280	-1.490429
1	-0.790592	0.681909	-2.281240
1	-0.595397	1.626865	-0.218667

4-Acetylbenzeneboronic acid and 1-Butylamine

Z-Isomer

E

6	1.358824	1.453834	1.940854
6	1.507769	1.405888	0.449182
8	0.665772	1.927457	-0.278102
6	2.658462	0.689369	-0.170357
6	2.833814	0.791295	-1.556095
6	3.885686	0.137291	-2.178114
6	4.785907	-0.647301	-1.441688
6	3.549962	-0.090639	0.574528
6	4.594624	-0.752863	-0.058301
5	5.971917	-1.386760	-2.146457
7	-1.419602	-0.296510	2.042876
6	-1.669751	-1.006107	0.796370
6	-3.306683	-1.662651	-1.052256
6	-3.098669	-0.916022	0.260968
6	-4.731105	-1.560992	-1.582897
8	6.097566	-1.241164	-3.500630
8	6.811215	-2.133351	-1.365617
1	2.310334	1.417927	2.473059
1	0.726216	0.603076	2.246028
1	0.814686	2.360825	2.211947
1	2.128306	1.394133	-2.118975
1	4.020410	0.226997	-3.252360
1	3.421385	-0.196719	1.646983
1	5.278647	-1.36293 0	0.524671
1	-2.032355	-0.65522 0	2.769389
1	-0.981553	-0.605535	0.040457
1	-1.393764	-2.061186	0.928652
1	-3.041729	-2.719608	-0.913059
1	-2.605808	-1.273798	-1.803238
1	-3.352538	0.14561 0	0.130226
1	-3.794745	-1.305131	1.019002
1	-4.850583	-2.103057	-2.526275
1	-5.012353	-0.517163	-1.761354
1	-5.449937	-1.976052	-0.867713
1	6.836215	-1.709519	-3.896963
1	7.525677	-2.571214	-1.834508
1	-1.6596 00	0.68814 0	1.908190
8	-1.697916	2.563938	1.070176
1	-2.38844 0	2.75538 0	0.430511
1	-0.889971	2.441734	0.535917

TS_{E-F}

6	1.280312	1.794370	2.175564
6	0.867322	1.349395	0.766036
8	0.422009	2.358820	0.006416
6	1.960287	0.531420	0.086317
6	2.427822	0.941879	-1.160735
6	3.431056	0.225783	-1.804948
6	3.990840	-0.922233	-1.229800

6	2.514848	-0.609639	0.675396
6	3.510619	-1.326564	0.023900
5	5.114523	-1.725043	-1.956207
7	-0.381261	0.400255	1.001787
6	-0.852716	-0.282443	-0.214133
6	-2.752243	-1.566075	-1.283875
6	-2.233471	-0.888677	-0.019022
6	-4.133634	-2.180452	-1.099987
8	5.546631	-1.270103	-3.174021
8	5.608372	-2.844306	-1.337464
1	2.122677	2.481418	2.071580
1	1.591405	0.967923	2.824094
1	0.450193	2.333608	2.641109
1	1.983730	1.831503	-1.595167
1	3.791539	0.557729	-2.775067
1	2.174218	-0.945048	1.653706
1	3.931511	-2.213114	0.490791
1	-0.220030	-0.257561	1.761769
1	-0.860852	0.489367	-0.990229
1	-0.122178	-1.042443	-0.508985
1	-2.041372	-2.341898	-1.597063
1	-2.779044	-0.832426	-2.099871
1	-2.933231	-0.102378	0.294079
1	-2.206370	-1.621619	0.800268
1	-4.483543	-2.658122	-2.019796
1	-4.870684	-1.420625	-0.818531
1	-4.127033	-2.941382	-0.311983
1	6.240446	-1.793989	-3.581391
1	6.300382	-3.302619	-1.820127
1	-1.136705	1.200955	1.261142
8	-1.615372	2.553880	1.228249
1	-2.384418	2.702768	0.670861
1	-0.628297	2.669087	0.560365
F			
6	1.290296	1.731695	2.092698
6	0.828437	1.235309	0.720284
8	0.469467	2.314201	-0.095139
6	1.956495	0.479303	0.022508
6	2.530512	0.964163	-1.152454
6	3.567159	0.269159	-1.767361
6	4.061987	-0.927308	-1.235099
6	2.442434	-0.715567	0.563483
6	3.475793	-1.406199	-0.055052
5	5.224672	-1.702908	-1.927392
7	-0.377783	0.400446	0.931534
6	-0.879655	-0.270231	-0.267013
6	-2.812804	-1.594196	-1.244697
6	-2.243558	-0.895368	-0.014383
6	-4.178627	-2.220087	-0.994399
8	5.755239	-1.178902	-3.077007
8	5.654226	-2.869293	-1.348832
1	2.149633	2.392428	1.961072
1	1.589823	0.907489	2.747877
1	0.482120	2.291168	2.573742
1	2.152813	1.889722	-1.571766
1	4.005683	0.659798	-2.681897
1	2.009911	-1.114837	1.479244
1	3.840360	-2.333362	0.379097
1	-0.193719	-0.283362	1.660557
1	-0.949749	0.493786	-1.048768
1	-0.178918	-1.034987	-0.636276
1	-2.108871	-2.367133	-1.580020
1	-2.883167	-0.872621	-2.069394
1	-2.941377	-0.118610	0.327766
1	-2.169590	-1.618973	0.810848
1	-4.563521	-2.714771	-1.891249
1	-4.911494	-1.464155	-0.691493
1	-4.130428	-2.969310	-0.196543
1	6.468250	-1.691184	-3.465540
1	6.372746	-3.309541	-1.808996
1	-1.536998	1.762542	1.402254
8	-1.813903	2.711224	1.425481
1	-2.588705	2.763648	0.858570
1	-0.274901	2.758224	0.358779
G			
6	1.578734	0.298672	0.742606
6	0.796473	-0.081543	-0.517938
6	1.621837	0.207047	-1.768689
6	2.806775	-0.505823	-1.983833
6	3.601717	-0.234334	-3.088286
6	3.244337	0.754445	-4.017084
6	1.247969	1.180843	-2.693822
6	2.050658	1.450173	-3.798948
7	0.460915	-1.465709	-0.440309
6	-0.212878	-2.109172	-1.558247
6	-2.332084	-2.621568	-2.859956
6	-1.693414	-1.778388	-1.761911
6	-3.809233	-2.312637	-3.068537
5	4.142544	1.059813	-5.256003
8	3.731977	2.038211	-6.123289
8	5.294106	0.333465	-5.409443
1	1.838788	1.362560	0.730791
1	2.504779	-0.275037	0.807272
1	0.968577	0.091257	1.627334
1	3.084574	-1.288978	-1.284002
1	4.518369	-0.797143	-3.242930
1	0.312105	1.713680	-2.559543
1	1.744966	2.211195	-4.511966

1	0.345583	-1.877864	-2.472511
1	-0.112484	-3.192810	-1.408129
1	-2.209949	-3.686856	-2.620123
1	-1.787968	-2.460800	-3.800546
1	-1.801336	-0.712984	-1.993962
1	-2.226205	-1.942787	-0.814005
1	-4.243069	-2.929378	-3.861905
1	-3.957501	-1.262808	-3.344284
1	-4.383985	-2.496172	-2.153765
1	4.316231	2.189482	-6.870035
1	5.814524	0.554352	-6.185505
8	-0.420493	0.724355	-0.528492
1	-0.167248	1.639792	-0.358398
1	-0.037792	-1.634374	0.430797
8	-1.592381	-0.574789	1.671763
1	-2.478441	-0.902989	1.497504
1	-1.386392	-0.003918	0.910625

TS_{G-H}

6	1.576764	0.255351	0.872218
6	1.030198	-0.347349	-0.386982
6	1.722520	0.005262	-1.661894
6	2.962245	-0.591259	-1.922415
6	3.653982	-0.278474	-3.085759
6	3.135526	0.634438	-4.013390
6	1.189565	0.917774	-2.574247
6	1.895051	1.221517	-3.733612
7	0.321713	-1.441334	-0.281274
6	-0.201606	-2.169783	-1.425620
6	-2.166940	-2.512419	-2.983982
6	-1.634740	-1.765132	-1.766692
6	-3.604529	-2.138365	-3.321598
5	3.917927	0.985086	-5.319328
8	3.353802	1.887029	-6.180778
8	5.123441	0.369601	-5.527974
1	1.775604	1.318086	0.731920
1	2.532158	-0.236144	1.098487
1	0.887873	0.099810	1.704320
1	3.379302	-1.304456	-1.215698
1	4.613988	-0.747473	-3.283016
1	0.227876	1.362492	-2.340645
1	1.476357	1.931165	-4.442114
1	0.453425	-2.008688	-2.287388
1	-0.163104	-3.238753	-1.182005
1	-2.100528	-3.595463	-2.810585
1	-1.521061	-2.303333	-3.847382
1	-1.664598	-0.681365	-1.927132
1	-2.278282	-1.960191	-0.899238
1	-3.963404	-2.678144	-4.203330
1	-3.694219	-1.066200	-3.525748
1	-4.279059	-2.372209	-2.490689
1	3.866729	2.074497	-6.970480
1	5.570312	0.613686	-6.341991
8	-0.719820	1.018306	-0.362417
1	-0.541987	1.892069	0.001209
1	-0.357814	-1.359714	0.582381
8	-1.301489	-0.518645	1.390828
1	-2.217345	-0.809051	1.375090
1	-1.128145	0.336254	0.539248

H

6	1.658999	0.176358	0.824701
6	1.252911	-0.598590	-0.392650
6	1.943598	-0.211548	-1.662900
6	3.128721	-0.837231	-2.058787
6	3.766178	-0.442482	-3.229198
6	3.243923	0.583610	-4.027600
6	1.409325	0.819742	-2.442952
6	2.057685	1.203424	-3.611552
7	0.330259	-1.476532	-0.297139
6	-0.148166	-2.182980	-1.471082
6	-2.154254	-2.534758	-2.985885
6	-1.604407	-1.820237	-1.756505
6	-3.611381	-2.191136	-3.267121
5	3.965058	1.024748	-5.340071
8	3.399918	2.031780	-6.076742
8	5.124496	0.380289	-5.683486
1	1.310338	1.208137	0.702480
1	2.748724	0.202659	0.924317
1	1.208731	-0.243435	1.726051
1	3.546050	-1.636179	-1.450767
1	4.685535	-0.934356	-3.535082
1	0.492632	1.304894	-2.113564
1	1.640089	2.002071	-4.218841
1	0.462740	-1.983498	-2.361676
1	-0.080327	-3.258700	-1.259818
1	-2.050434	-3.620589	-2.854995
1	-1.540733	-2.274209	-3.858312
1	-1.681991	-0.732742	-1.881912
1	-2.217103	-2.073242	-0.880231
1	-3.981104	-2.708068	-4.158024
1	-3.737884	-1.115249	-3.428255
1	-4.254647	-2.475932	-2.427080
1	3.877245	2.269602	-6.875185
1	5.533391	0.685393	-6.496883
8	-1.194020	1.548104	-0.395424
1	-1.407303	2.390808	0.012477
1	-0.849366	-1.025669	0.985255
8	-1.490396	-0.471874	1.497236
1	-2.331854	-0.930490	1.422718

1	-1.369488	0.884585	0.302782

E-Isomer

G'

6	1.788428	-0.226097	1.002696
6	0.802978	0.184370	-0.091552
6	1.562236	0.402261	-1.398995
6	1.776215	-0.666259	-2.273033
6	2.512752	-0.484576	-3.437879
6	3.058275	0.762935	-3.767988
6	2.106191	1.651799	-1.711919
6	2.838646	1.824921	-2.880954
7	-0.209785	-0.810537	-0.297125
6	-0.911931	-1.399364	0.827905
6	-2.673866	-1.260532	2.656362
6	-1.775457	-0.479462	1.702695
6	-3.515522	-0.367809	3.558852
5	3.881885	0.961347	-5.078437
8	4.386276	2.208429	-5.340003
8	4.057104	-0.122863	-5.898054
1	2.584634	0.517952	1.068433
1	2.231679	-1.197535	0.770518
1	1.299137	-0.292484	1.980864
1	1.344116	-1.631362	-2.028255
1	2.668600	-1.323506	-4.110871
1	1.942675	2.484336	-1.035816
1	3.251007	2.802708	-3.115605
1	-1.565931	-2.173841	0.407579
1	-0.194325	-1.935947	1.461156
1	-2.055628	-1.928656	3.271403
1	-3.331494	-1.914350	2.068269
1	-2.387788	0.163049	1.055750
1	-1.138251	0.188287	2.304906
1	-4.156021	-0.957736	4.221563
1	-4.163051	0.290717	2.969881
1	-2.884027	0.268318	4.188937
1	4.886120	2.281299	-6.156546
1	4.570032	0.046537	-6.691825
8	0.263359	1.490877	0.244622
1	-0.152082	1.411902	1.110063
1	-0.864303	-0.452966	-0.992580
8	-1.572731	1.344590	-1.906501
1	-0.976559	1.392686	-2.660512
1	-1.026374	1.670555	-1.171223

$TS_{G'-H'}$

6	1.755361	-0.023325	1.112943
6	0.927387	-0.070770	-0.137819
6	1.631666	0.257312	-1.415347
6	1.617884	-0.679607	-2.453523
6	2.324439	-0.439373	-3.626888
6	3.068382	0.733593	-3.796191
6	2.371304	1.435340	-1.573115
6	3.073819	1.664704	-2.746697
7	-0.131563	-0.850616	-0.254415
6	-0.797605	-1.446815	0.889207
6	-2.616179	-1.242756	2.647170
6	-1.766147	-0.509879	1.615491
6	-3.572066	-0.319766	3.392143
5	3.865665	0.998942	-5.113059
8	4.572583	2.167862	-5.210060
8	3.814403	0.047374	-6.096899
1	2.420578	0.839056	1.087995
1	2.374893	-0.926718	1.164968
1	1.142173	0.026543	2.014281
1	1.058175	-1.600205	-2.321388
1	2.306808	-1.175834	-4.425594
1	2.334785	2.185917	-0.791396
1	3.634668	2.587693	-2.864802
1	-1.363973	-2.305412	0.508576
1	-0.059435	-1.853810	1.591420
1	-1.961700	-1.753637	3.366957
1	-3.187877	-2.033822	2.143783
1	-2.405674	-0.022281	0.869404
1	-1.203456	0.290412	2.116093
1	-4.177622	-0.870781	4.118211
1	-4.255792	0.181073	2.698610
1	-3.027583	0.459017	3.937468
1	5.045266	2.291041	-6.036695
1	4.318261	0.252409	-6.888103
8	0.077316	1.828918	-0.003639
1	-0.350901	1.895383	0.857206
1	-0.849371	-0.328131	-0.998937
8	-1.551630	0.772499	-1.419779
1	-1.407008	0.960366	-2.352206
1	-0.765014	1.456076	-0.738591

H'

6	1.922625	-0.223567	1.258531
6	1.204784	-0.586394	-0.012028
6	1.822833	-0.133965	-1.289332
6	1.621627	-0.875604	-2.461348
6	2.174104	-0.455567	-3.663450
6	2.938598	0.718134	-3.739934
6	2.589508	1.035183	-1.353105
6	3.129388	1.453851	-2.563326
7	0.080626	-1.193989	-0.108885
6	-0.647421	-1.652355	1.053792
6	-2.549716	-1.108104	2.647785
6	-1.758202	-0.666356	1.422459
6	-3.665908	-0.136214	3.009230

5	3.558885	1.188514	-5.094353
8	4.300575	2.339400	-5.094149
8	3.324385	0.423167	-6.205488
1	3.004803	-0.216169	1.112857
1	1.683976	-0.898114	2.081891
1	1.598707	0.785738	1.540343
1	1.032318	-1.785804	-2.403241
1	2.020501	-1.045442	-4.563101
1	2.724149	1.641535	-0.463288
1	3.708244	2.372368	-2.603513
1	-1.097994	-2.619803	0.797283
1	-0.007658	-1.822119	1.931706
1	-1.868717	-1.218650	3.502555
1	-2.974634	-2.105075	2.468278
1	-2.433232	-0.554749	0.563995
1	-1.309325	0.321853	1.584307
1	-4.221901	-0.471211	3.890369
1	-4.379206	-0.030608	2.184719
1	-3.265362	0.859879	3.227087
1	4.660804	2.593196	-5.947225
1	3.724650	0.746995	-7.015961
8	-0.105737	2.254685	0.360053
1	-0.796932	2.821324	0.711825
1	-0.923923	-0.110382	-1.313033
8	-1.265846	0.726126	-1.694310
1	-0.705697	0.866962	-2.464413
1	-0.535458	1.793323	-0.388796

2-Acetylbenzeneboronic acid and 1-Butylamine

Hypothetic Mechanism 1

Z-Isomer

I

6	0.854547	-1.945101	-1.003922
6	1.269959	-1.477102	0.349058
8	0.731053	-1.914872	1.367853
6	2.293517	-0.418412	0.520219
7	-1.246787	0.546246	-1.240847
6	-0.743351	1.913531	-1.265647
6	2.482318	0.009713	1.845675
6	3.953076	1.138222	-0.272620
6	3.017303	0.144188	-0.534165
6	-1.706411	2.979761	-0.744953
6	-1.122982	4.388638	-0.770494
6	-2.089323	5.447255	-0.254348
6	3.428260	1.006794	2.084749
5	1.620464	-0.640238	3.008539
6	4.159295	1.565752	1.037114
8	2.113338	-1.596428	3.860739
8	0.486264	0.028446	3.425675
1	1.700669	-2.063483	-1.684998
1	0.175746	-1.169005	-1.409571
1	0.309990	-2.885903	-0.913649
1	-1.341941	-1.248237	1.475046
1	-2.103530	0.492804	-1.784736
1	0.180487	1.941293	-0.672557
1	-0.448089	2.161053	-2.294815
1	4.519257	1.580279	-1.086906
1	2.848891	-0.184205	-1.555101
1	-2.002836	2.719185	0.280551
1	-2.628813	2.954517	-1.344203
1	-0.819803	4.637432	-1.796870
1	-0.202565	4.408243	-0.171339
1	-1.644808	6.447204	-0.280480
1	-2.384095	5.240809	0.780324
1	-3.004160	5.474258	-0.856670
1	3.594849	1.363337	3.098264
1	4.889777	2.343552	1.242299
1	2.910449	-2.011761	3.525136
1	0.127729	-0.406235	4.207761
8	-1.716560	-0.370531	1.615572
1	-1.044248	0.034123	2.185534
1	-1.499593	0.297473	-0.279536

TS_{I-J}

6	0.786343	-2.089774	-1.043142
6	1.093575	-1.270367	0.164994
8	0.589955	-1.566080	1.267874
6	2.157901	-0.256886	0.213497
7	-0.694119	0.491333	-0.669536
6	-0.329427	1.888800	-0.479667
6	2.347100	0.193141	1.526439
6	3.897761	1.167390	-0.606321
6	2.917107	0.214726	-0.857202
6	-1.491702	2.878255	-0.550223
6	-1.062775	4.325474	-0.331705
6	-2.221818	5.310917	-0.412040
6	3.332362	1.151747	1.753873
5	1.388215	-0.522827	2.593434
6	4.101255	1.633366	0.694288
8	1.908368	-1.441323	3.506573
8	0.320817	0.234205	3.118165
1	1.505638	-2.917669	-1.087539
1	0.869476	-1.504657	-1.958194
1	-0.215311	-2.512328	-0.961419
1	-1.721228	-1.333196	1.641041
1	-1.186359	0.382542	-1.552251
1	0.161525	1.972315	0.497810
1	0.431757	2.159395	-1.224600

1	4.504084	1.551947	-1.421097
1	2.748641	-0.146223	-1.867992
1	-2.243523	2.596445	0.199697
1	-1.986089	2.787437	-1.529210
1	-0.299675	4.593914	-1.075164
1	-0.573220	4.411463	0.647442
1	-1.888040	6.340650	-0.249988
1	-2.983903	5.085587	0.341903
1	-2.708347	5.269465	-1.392912
1	3.506553	1.529347	2.758403
1	4.867922	2.381201	0.879327
1	2.732978	-1.827593	3.206536
1	0.108492	-0.127792	3.985487
8	-2.060817	-0.443175	1.786761
1	-1.316515	-0.036984	2.269068
1	-1.337612	0.204406	0.075272
J			
6	0.651167	-1.757932	-1.121987
6	0.755712	-0.695201	-0.042149
8	0.466717	-1.195810	1.181592
6	2.081013	0.030640	0.052578
7	-0.405057	0.358956	-0.379743
6	-0.150449	1.805705	-0.160460
6	2.395828	0.214130	1.400949
6	4.102118	1.095201	-0.653230
6	2.906018	0.458764	-0.982924
6	-1.407371	2.615719	-0.438967
6	-1.189125	4.104965	-0.186608
6	-2.434664	4.936105	-0.464521
6	3.594335	0.858418	1.705810
5	1.192605	-0.423156	2.301857
6	4.442077	1.292096	0.685760
8	1.529348	-1.254450	3.408641
8	0.228476	0.599576	2.784831
1	1.395220	-2.528512	-0.913010
1	0.842435	-1.356471	-2.122848
1	-0.337666	-2.227542	-1.101463
1	-2.026240	-1.033309	1.794926
1	-0.737392	0.214454	-1.332427
1	0.162728	1.907847	0.882619
1	0.681651	2.112092	-0.799760
1	4.773755	1.430134	-1.438899
1	2.645575	0.297705	-2.027790
1	-2.222310	2.246715	0.196662
1	-1.726506	2.467843	-1.481914
1	-0.358703	4.462974	-0.809290
1	-0.873890	4.248392	0.854446
1	-2.255663	5.997551	-0.269959
1	-3.270537	4.617939	0.167412
1	-2.752961	4.838735	-1.508283
1	3.880398	1.016642	2.743931
1	5.380502	1.783644	0.930668
1	2.263045	-1.828436	3.181173
1	0.324928	0.640286	3.740722
8	-2.078491	-0.075438	1.696291
1	-1.296263	0.218886	2.244762
1	-1.175015	0.088739	0.304282
TS_{J-K}			
6	0.634530	-1.624081	-1.180672
6	0.692558	-0.610051	-0.048408
8	0.462491	-1.248745	1.159819
6	2.033159	0.091791	0.095054
7	-0.452609	0.388114	-0.269174
6	-0.165380	1.834373	-0.241742
6	2.396981	0.128750	1.443112
6	4.048485	1.211712	-0.548040
6	2.837403	0.622080	-0.908977
6	-1.441344	2.632094	-0.471455
6	-1.199763	4.137726	-0.424295
6	-2.466163	4.946252	-0.673999
6	3.607393	0.729919	1.784261
5	1.241434	-0.631024	2.281070
6	4.429489	1.266434	0.792906
8	1.580865	-1.541351	3.323684
8	0.287022	0.369862	2.968374
1	1.402788	-2.381895	-1.017660
1	0.805187	-1.157817	-2.156700
1	-0.340180	-2.123983	-1.187405
1	-1.946351	-0.863482	1.885140
1	-0.918672	0.167573	-1.146445
1	0.259738	2.066064	0.740210
1	0.594490	2.098844	-0.986498
1	4.700320	1.623291	-1.313913
1	2.546951	0.574596	-1.957019
1	-2.188167	2.351356	0.282139
1	-1.870193	2.365921	-1.449576
1	-0.437148	4.407595	-1.166721
1	-0.778827	4.403419	0.553810
1	-2.268759	6.021382	-0.629377
1	-3.234634	4.717604	0.072184
1	-2.888493	4.726671	-1.660773
1	3.922835	0.772232	2.825011
1	5.378155	1.724838	1.061049
1	2.290548	-2.120724	3.041172
1	0.318725	0.165529	3.909686
8	-1.801550	0.088926	1.817174
1	-0.849148	0.240781	2.470496
1	-1.266819	0.186017	0.790178

K			
6	0.654656	-1.671355	-1.154619
6	0.684746	-0.577446	-0.096417
8	0.426245	-1.168949	1.155635
6	2.046350	0.087016	0.055094
7	-0.409446	0.391590	-0.399757
6	-0.154402	1.827102	-0.244635
6	2.413351	0.130446	1.402647
6	4.078458	1.179857	-0.584917
6	2.860197	0.605687	-0.946670
6	-1.426466	2.623291	-0.503132
6	-1.218908	4.126475	-0.349135
6	-2.485200	4.929273	-0.617811
6	3.631192	0.713479	1.747582
5	1.253115	-0.617403	2.215670
6	4.461427	1.234907	0.755428
8	1.523434	-1.471584	3.319652
8	0.278871	0.476579	2.964782
1	1.383043	-2.442720	-0.898712
1	0.893668	-1.279481	-2.148653
1	-0.338502	-2.131918	-1.182144
1	-2.040061	-0.865634	1.937127
1	-0.765757	0.219306	-1.334531
1	0.192609	2.003230	0.779598
1	0.650972	2.180712	-0.905227
1	4.735050	1.582855	-1.351358
1	2.567764	0.563057	-1.993997
1	-2.213998	2.284311	0.182121
1	-1.789579	2.411451	-1.520396
1	-0.421811	4.456130	-1.028647
1	-0.857895	4.337473	0.665928
1	-2.312627	6.002922	-0.496172
1	-3.288447	4.641262	0.068982
1	-2.849456	4.764780	-1.637933
1	3.944371	0.757693	2.788980
1	5.414962	1.683245	1.022373
1	2.306027	-1.999054	3.152002
1	0.289768	0.207256	3.892708
8	-1.949941	0.087142	1.814978
1	-0.675006	0.353411	2.586532
1	-1.509424	0.138198	0.908723
TS_{K-L}			
6	0.672685	-1.694840	-1.176854
6	0.693186	-0.574188	-0.146398
8	0.407986	-1.156117	1.122479
6	2.067663	0.060119	0.017252
7	-0.374495	0.385539	-0.475463
6	-0.146214	1.813838	-0.240121
6	2.474472	0.023429	1.353549
6	4.095695	1.165249	-0.611932
6	2.863451	0.624154	-0.974252
6	-1.422159	2.603433	-0.498850
6	-1.239432	4.100695	-0.273491
6	-2.509807	4.898106	-0.538983
6	3.706525	0.573495	1.701409
5	1.322768	-0.751637	2.106373
6	4.514662	1.142623	0.718998
8	1.427895	-1.539319	3.242625
8	0.216500	0.740861	2.939881
1	1.381090	-2.471916	-0.884993
1	0.945291	-1.327363	-2.171352
1	-0.327989	-2.136470	-1.220114
1	-2.026401	-0.887179	1.922820
1	-0.681575	0.244043	-1.431744
1	0.165965	1.942737	0.801509
1	0.671540	2.212164	-0.860039
1	4.735775	1.606849	-1.370791
1	2.542343	0.645171	-2.013526
1	-2.221382	2.224376	0.150328
1	-1.755516	2.432060	-1.533923
1	-0.430088	4.471087	-0.916845
1	-0.907743	4.270146	0.759124
1	-2.355828	5.967519	-0.365542
1	-3.325555	4.568936	0.113651
1	-2.846282	4.774462	-1.574373
1	4.049678	0.563675	2.734526
1	5.477421	1.569909	0.985780
1	2.285362	-1.422942	3.656321
1	0.190834	0.418673	3.847241
8	-2.068388	0.053294	1.715689
1	-0.688791	0.551032	2.568689
1	-1.597790	0.088891	0.843024
L			
6	0.685790	-1.801225	-1.031972
6	0.790343	-0.554478	-0.167303
8	0.496824	-0.928730	1.189311
6	2.207413	0.003524	-0.104375
7	-0.231864	0.394749	-0.578106
6	-0.097341	1.772731	-0.097341
6	2.678671	0.034287	1.212209
6	4.259206	0.948714	-0.887140
6	2.984229	0.459411	-1.163095
6	-1.324658	2.591785	-0.469098
6	-1.269239	4.001270	0.111590
6	-2.485271	4.840525	-0.257741
6	3.956957	0.526592	1.475126
5	1.506144	-0.566633	2.043318

6	4.744605	0.982595	0.422197
8	1.336628	-0.785390	3.377128
8	-0.906451	1.356397	3.085560
1	1.368658	-2.568566	-0.662315
1	0.945835	-1.580317	-2.071818
1	-0.336958	-2.185889	-0.995153
1	-1.925940	-1.083757	1.642738
1	-0.332550	0.374685	-1.587687
1	-0.020262	1.737387	0.995506
1	0.811640	2.259743	-0.483995
1	4.884209	1.309332	-1.699312
1	2.614377	0.441737	-2.185652
1	-2.222080	2.081134	-0.098304
1	-1.424078	2.646152	-1.564223
1	-0.355566	4.504372	-0.233303
1	-1.186670	3.924745	1.202927
1	-2.426387	5.843551	0.175865
1	-3.408516	4.375930	0.104843
1	-2.576778	4.952586	-1.344135
1	4.347396	0.558990	2.490107
1	5.741537	1.368040	0.614814
1	2.090473	-0.490841	3.893512
1	-0.390435	0.664254	3.512539
8	-2.334506	-0.300133	1.259931
1	-1.528635	0.859456	2.520265
1	-1.729397	-0.108122	0.512262

M

6	0.731939	-1.771278	-1.041925
6	0.859929	-0.481767	-0.245891
8	0.541006	-0.834070	1.158248
6	2.286613	0.025805	-0.159325
7	-0.031728	0.499424	-0.757327
6	-0.060622	1.789629	-0.065739
6	2.771751	0.006558	1.152144
6	4.365088	0.898252	-0.948124
6	3.065876	0.475875	-1.218163
6	-1.267861	2.600835	-0.507807
6	-1.345514	3.950199	0.199132
6	-2.568570	4.761973	-0.206704
6	4.075256	0.434684	1.408207
5	1.576185	-0.515329	1.999335
6	4.869910	0.875544	0.354868
8	1.399245	-0.679929	3.342378
1	1.389920	-2.540340	-0.631890
1	0.992972	-1.593195	-2.087231
1	-0.299615	-2.135099	-0.995917
1	-0.971236	0.112193	-0.786983
1	-0.099890	1.678224	1.028046
1	0.861385	2.332023	-0.309087
1	4.994150	1.254473	-1.759155
1	2.665979	0.509409	-2.227653
1	-2.178228	2.027119	-0.281954
1	-1.241141	2.740412	-1.597031
1	-0.434855	4.525713	-0.015610
1	-1.352628	3.780756	1.283367
1	-2.602029	5.724175	0.313693
1	-3.493809	4.225043	0.029662
1	-2.571711	4.965543	-1.283507
1	4.477751	0.431110	2.419052
1	5.886026	1.210165	0.542571
1	2.171758	-0.412687	3.846417
8	-0.915608	1.397776	3.335593
1	-0.283303	0.729893	3.623741
1	-1.525439	0.897520	2.768552
8	-2.165605	-0.402832	1.477495
1	-2.660581	-1.104538	1.909376
1	-1.244893	-0.720185	1.433352

TS_{M-N}

6	0.544730	-1.682670	-1.154996
6	0.782186	-0.326844	-0.583583
8	0.609137	-1.390588	1.740237
6	2.180841	0.091395	-0.304585
7	-0.227139	0.431283	-0.320286
6	-0.145170	1.791569	0.199568
6	2.586687	0.017332	1.047154
6	4.362097	0.848184	-0.988150
6	3.054563	0.493115	-1.312329
6	-1.261429	2.645924	-0.387864
6	-1.298139	4.031885	0.247392
6	-2.412232	4.906599	-0.311951
6	3.907000	0.376470	1.331960
5	1.525013	-0.513840	2.127023
6	4.789266	0.786602	0.334175
8	1.569996	-0.013329	3.421670
1	1.143777	-2.417155	-0.615914
1	0.858653	-1.681491	-2.206552
1	-0.511032	-1.953521	-1.099926
1	-1.112240	-0.133291	0.089105
1	-0.280714	1.739048	1.291353
1	0.839505	2.212958	-0.022563
1	5.044701	1.167980	-1.769866
1	2.716916	0.541815	-2.344508
1	-2.220326	2.141200	-0.211338
1	-1.141984	2.727677	-1.476585
1	-0.329930	4.528580	0.096878
1	-1.421064	3.916720	1.331418
1	-2.419037	5.893839	0.159615
1	-3.393760	4.451232	-0.141612

1	-2.299077	5.054417	-1.391787
1	4.264731	0.314040	2.358431
1	5.811915	1.052436	0.586423
1	2.220814	0.687408	3.516112
8	-1.177831	1.173283	3.165635
1	-0.398390	0.751319	3.551256
1	-1.570691	0.463828	2.624032
8	-1.650850	-0.796954	1.241718
1	-2.288831	-1.510491	1.158280
1	-0.512788	-1.200886	1.621164

N

6	0.641713	-1.661333	-1.386884
6	0.802848	-0.320544	-0.737454
8	0.830478	-1.564489	1.814170
6	2.205974	0.069899	-0.376114
7	-0.235233	0.375018	-0.472144
6	-0.129510	1.694891	0.131373
6	2.607336	0.007155	0.975549
6	4.402301	0.855452	-1.014929
6	3.104537	0.485196	-1.358688
6	-1.219311	2.611487	-0.411543
6	-1.261667	3.948587	0.319798
6	-2.347149	4.878491	-0.206138
6	3.914807	0.389664	1.294674
5	1.577931	-0.465660	2.070894
6	4.810813	0.806287	0.313867
8	1.405871	0.225947	3.248384
1	0.965704	-2.440848	-0.689927
1	1.272490	-1.735906	-2.279789
1	-0.401501	-1.824902	-1.663838
1	-1.455237	-0.523568	0.631123
1	-0.291775	1.599724	1.215283
1	0.861401	2.146658	-0.018804
1	5.093223	1.182387	-1.786746
1	2.784650	0.532058	-2.396660
1	-2.185722	2.102163	-0.302699
1	-1.071117	2.766982	-1.488781
1	-0.283882	4.442696	0.234436
1	-1.418206	3.755886	1.388863
1	-2.359951	5.828316	0.337389
1	-3.338900	4.424638	-0.102587
1	-2.197805	5.104626	-1.267974
1	4.247675	0.342136	2.330103
1	5.824065	1.088906	0.584223
1	1.922398	1.037204	3.258627
8	-1.384973	1.349364	3.239187
1	-0.549377	0.992936	3.563839
1	-1.723062	0.624772	2.686955
8	-1.847386	-0.845978	1.474537
1	-2.745248	-1.116049	1.264944
1	-0.017330	-1.605171	2.277684

***E*-Isomer**

M'

6	1.811138	-2.261035	-1.113255
6	1.506106	-0.853818	-0.617818
8	0.505847	-1.020623	0.470672
6	2.692296	-0.227641	0.092565
7	1.042879	0.009159	-1.642786
6	2.463517	-0.071340	1.462834
6	4.872575	0.720542	0.318604
6	3.884960	0.173628	-0.496762
6	3.461139	0.481718	2.268164
5	1.004802	-0.570659	1.660172
6	4.666870	0.870429	1.693110
8	0.189171	-0.575980	2.760519
6	-0.085907	-0.433009	-2.449591
6	-0.539504	0.692513	-3.368995
6	-1.710456	0.296174	-4.260568
6	-2.163054	1.424679	-5.178424
1	0.895609	-2.761645	-1.436223
1	2.256489	-2.849428	-0.308138
1	2.510515	-2.218994	-1.952089
1	0.830604	0.923557	-1.233426
1	5.814028	1.041528	-0.118657
1	4.031232	0.076897	-1.568411
1	3.309117	0.616146	3.337555
1	5.451102	1.300332	2.309607
1	0.643040	-0.263426	3.546690
1	0.218183	-1.293427	-3.059021
1	-0.942687	-0.768725	-1.838238
1	0.313049	1.006757	-3.985113
1	-0.816098	1.562207	-2.757836
1	-2.552236	-0.025909	-3.633191
1	-1.430194	-0.577739	-4.864604
1	-3.003155	1.118071	-5.809355
1	-1.350370	1.746259	-5.839037
1	-2.481830	2.298571	-4.600134
8	-0.110476	2.320441	-0.145824
1	0.434433	2.652611	0.572066
1	-0.814038	1.793211	0.286253
8	-1.876057	0.468169	0.922841
1	-1.679001	0.305443	1.853520
1	-1.353834	-0.227722	0.494130

$TS_{M'-N'}$

6	2.160586	-1.996120	-1.715764
6	1.858114	-0.685183	-1.058330
8	0.096147	-1.049949	1.244741

6	2.810090	-0.222076	-0.014003
7	0.813843	0.002773	-1.343897
6	2.400250	0.029050	1.311300
6	5.106329	0.327022	0.531298
6	4.151745	-0.082535	-0.390674
6	3.394689	0.418879	2.218196
5	0.872970	-0.119669	1.765214
6	4.727129	0.567622	1.848373
8	0.402001	0.838142	2.667482
6	-0.258755	-0.400349	-2.243696
6	-0.565899	0.705892	-3.247120
6	-1.775232	0.366411	-4.111959
6	-2.100654	1.460394	-5.120359
1	1.564967	-2.758407	-1.198379
1	3.210478	-2.258910	-1.588622
1	1.903292	-2.018536	-2.776555
1	0.454788	0.892190	-0.730203
1	6.140759	0.447714	0.224016
1	4.449475	-0.273050	-1.419118
1	3.119105	0.589234	3.257825
1	5.468960	0.865394	2.584070
1	1.094106	1.456716	2.916066
1	-0.018455	-1.337905	-2.749959
1	-1.130059	-0.574734	-1.596111
1	0.312599	0.880991	-3.882858
1	-0.750584	1.635038	-2.694813
1	-2.641566	0.193623	-3.460988
1	-1.596445	-0.579860	-4.640888
1	-2.974346	1.199027	-5.724885
1	-1.262351	1.632763	-5.804423
1	-2.315641	2.408415	-4.616167
8	-0.440737	1.777476	-0.095769
1	-0.108617	2.055547	0.765449
1	-1.228148	1.013765	0.178092
8	-1.928614	-0.050226	0.456803
1	-2.595979	0.139881	1.121681
1	-0.950538	-0.668525	0.988740

N'(1)

6	2.182197	-1.984048	-1.882835
6	1.860090	-0.716984	-1.120774
8	0.169602	-1.032769	1.487651
6	2.853362	-0.290885	-0.091147
7	0.802913	-0.018640	-1.283625
6	2.458661	0.062676	1.214325
6	5.170404	0.187442	0.457332
6	4.204646	-0.232606	-0.449906
6	3.453527	0.474339	2.111682
5	0.956383	0.030407	1.698934
6	4.795418	0.536063	1.750430
8	0.475745	1.141783	2.388829
6	-0.215326	-0.415475	-2.238036
6	-0.474559	0.703132	-3.244574
6	-1.606124	0.372726	-4.211952
6	-1.862276	1.485018	-5.220543
1	1.471150	-2.767469	-1.599079
1	3.180943	-2.347825	-1.641473
1	2.110580	-1.846203	-2.965758
1	-0.228198	1.301181	-0.444987
1	6.212113	0.239880	0.154298
1	4.503839	-0.495185	-1.461486
1	3.173305	0.730658	3.132518
1	5.543301	0.850148	2.472888
1	1.168691	1.803886	2.473569
1	0.016093	-1.347367	-2.769531
1	-1.132331	-0.600190	-1.660879
1	0.449317	0.899708	-3.805378
1	-0.709869	1.625324	-2.699290
1	-2.522733	0.176161	-3.640371
1	-1.374390	-0.559893	-4.744346
1	-2.679271	1.228191	-5.901724
1	-0.971833	1.681510	-5.827849
1	-2.129910	2.419675	-4.716243
8	-0.962119	1.795662	-0.012915
1	-0.589662	1.999979	0.855640
1	-1.995371	0.530240	0.654215
8	-2.348731	-0.218195	1.191287
1	-2.774030	0.199600	1.945452
1	-0.795742	-0.843729	1.581459

$TS_{N'-O}$

6	2.419365	-2.166576	-1.900507
6	2.138719	-0.874744	-1.170173
8	-0.146517	-0.518936	1.110974
6	2.950424	-0.593333	0.046183
7	1.243728	-0.035307	-1.536533
6	2.371517	-0.032776	1.200953
6	5.127288	-0.613451	1.127480
6	4.320405	-0.880759	0.027571
6	3.205396	0.222863	2.298034
5	0.846210	0.351872	1.321866
6	4.566308	-0.066116	2.275807
8	0.568390	1.646544	1.777525
6	0.463411	-0.373089	-2.716622
6	-0.644652	0.629476	-2.994943
6	-1.422852	0.305956	-4.265823
6	-2.550045	1.293712	-4.538092
1	3.197446	-2.755400	-1.415681
1	2.707249	-1.984375	-2.942419
1	1.508313	-2.775421	-1.927544

1	-0.267452	2.145013	-0.995385
1	6.190102	-0.833682	1.085842
1	4.770616	-1.297015	-0.869692
1	2.772147	0.638762	3.206457
1	5.182926	0.132859	3.147691
1	1.390259	2.132326	1.899110
1	1.123888	-0.433243	-3.597320
1	0.014744	-1.373535	-2.610624
1	-0.210071	1.635389	-3.083483
1	-1.334971	0.644807	-2.142634
1	-1.835738	-0.708819	-4.187355
1	-0.732890	0.287395	-5.120559
1	-3.093771	1.039697	-5.453136
1	-2.164277	2.312649	-4.651370
1	-3.270248	1.304968	-3.713252
8	-0.818968	2.646349	-0.380160
1	-0.335902	2.543365	0.458830
1	-2.031305	1.487485	0.159690
8	-2.469419	0.727847	0.608236
1	-2.943366	1.113808	1.350108
1	-1.048608	-0.118637	1.068754

O

6	2.800885	-1.793727	-2.357151
6	2.303231	-1.022075	-1.181013
8	-0.104734	-0.468433	1.173679
6	3.154970	-0.756241	-0.010832
7	1.118115	-0.538541	-1.030913
6	2.428602	-0.095091	0.992878
6	5.132369	-0.792042	1.335085
6	4.494385	-1.112000	0.139916
6	3.089771	0.216502	2.175672
5	0.917790	0.200613	0.495346
6	4.431750	-0.132876	2.345572
8	0.648674	1.621214	0.239111
6	0.001622	-0.701418	-1.937621
6	-0.275086	0.561613	-2.751474
6	-1.584138	0.456179	-3.526195
6	-1.883274	1.701428	-4.350603
1	3.741947	-1.358881	-2.707590
1	2.096318	-1.809508	-3.188309
1	3.017371	-2.824960	-2.057770
1	-1.755083	3.196584	-0.735087
1	6.175267	-1.057440	1.481781
1	5.037395	-1.628595	-0.647845
1	2.559910	0.723756	2.978271
1	4.939277	0.106809	3.276534
1	1.466344	2.113397	0.140651
1	0.149657	-1.561931	-2.600002
1	-0.869573	-0.915335	-1.308766
1	0.562559	0.752721	-3.436957
1	-0.316121	1.408041	-2.057861
1	-2.395981	0.276533	-2.810409
1	-1.552565	-0.420955	-4.187129
1	-2.828148	1.605044	-4.893828
1	-1.094315	1.893998	-5.086155
1	-1.959758	2.589403	-3.712496
8	-1.762910	2.754775	0.118322
1	-0.837061	2.433901	0.246386
1	-2.433507	1.185592	0.004054
8	-2.600103	0.213243	-0.024256
1	-3.293622	0.056593	0.622437
1	-0.986428	-0.239960	0.828369

Hypothetic Mechanism 2
Z-Isomer

P

6	0.748629	-1.662019	-1.033026
6	1.168646	-1.045013	0.259097
8	0.367694	-0.956675	1.194201
6	2.513694	-0.455884	0.458456
7	-1.265135	0.750338	-1.772604
6	-0.744385	1.967521	-1.157377
6	2.667101	0.318833	1.624670
6	4.817782	-0.077889	-0.150936
6	3.579930	-0.647002	-0.425091
6	-1.793929	2.913202	-0.576001
6	-1.180732	4.127311	0.114278
6	-2.220943	5.062178	0.718509
6	3.918998	0.879940	1.877636
5	1.433879	0.560606	2.596057
6	4.986134	0.684618	1.001750
8	1.311054	-0.069264	3.809730
8	0.635630	1.643245	2.350604
1	1.575173	-2.040917	-1.634328
1	0.210223	-0.875917	-1.600611
1	0.034729	-2.464347	-0.834924
1	-1.477033	-0.801373	0.949598
1	-1.879389	0.996589	-2.543622
1	-0.060754	1.675543	-0.350852
1	-0.134226	2.500580	-1.899108
1	5.648794	-0.228747	-0.833307
1	3.454020	-1.243298	-1.323084
1	-2.407331	2.350310	0.141231
1	-2.475503	3.243177	-1.374758
1	-0.564172	4.682307	-0.606346
1	-0.492992	3.777833	0.895260
1	-1.754765	5.920224	1.213540
1	-2.833702	4.541402	1.462890
1	-2.899825	5.450457	-0.049136

1	4.066988	1.489137	2.766006
1	5.952169	1.133023	1.217204
1	1.894731	-0.826942	3.886546
1	-0.059331	1.704332	3.014311
8	-2.396612	-0.644017	0.673451
1	-1.839928	0.262666	-1.081155
1	-2.765967	-1.523478	0.558924

TS_{P-Q}

6	0.522179	-1.954917	-0.802181
6	0.870979	-0.909874	0.218048
8	0.361513	-1.001275	1.372820
6	2.150884	-0.162153	0.183932
7	-0.403796	0.594310	-0.724154
6	-0.261595	1.917785	-0.123650
6	2.463966	0.356458	1.445998
6	4.157412	0.740432	-0.766928
6	2.979558	0.018010	-0.923052
6	-1.364841	2.899160	-0.508672
6	-1.176599	4.270660	0.132826
6	-2.284229	5.252722	-0.226024
6	3.649185	1.080623	1.577824
5	1.390902	0.016305	2.590627
6	4.488883	1.270308	0.481517
8	1.685652	-0.905685	3.600180
8	0.559895	1.063698	2.987072
1	1.069593	-2.869977	-0.547458
1	0.796845	-1.662860	-1.816962
1	-0.547107	-2.168421	-0.752998
1	-1.477431	-1.073716	1.264878
1	-0.350972	0.644656	-1.737690
1	-0.236018	1.783131	0.963873
1	0.718426	2.315619	-0.413268
1	4.819455	0.891695	-1.614741
1	2.717557	-0.393709	-1.894573
1	-2.335573	2.481875	-0.206930
1	-1.401252	3.008145	-1.603105
1	-0.205305	4.682217	-0.172340
1	-1.124629	4.151535	1.222509
1	-2.126410	6.226466	0.247801
1	-3.262212	4.880169	0.097782
1	-2.335889	5.414608	-1.308563
1	3.922957	1.501442	2.542307
1	5.410667	1.834821	0.595862
1	2.318176	-1.563607	3.306264
1	0.111993	0.810073	3.799848
8	-2.337023	-0.952558	0.818871
1	-1.299035	0.171162	-0.465312
1	-2.755417	-1.816738	0.835445

Q

6	0.522403	-1.794922	-0.783026
6	0.722359	-0.546508	0.066325
8	0.311445	-0.723008	1.343498
6	2.128945	0.007094	0.085455
7	-0.243464	0.532599	-0.577460
6	-0.158355	1.902368	-0.003395
6	2.498142	0.317091	1.394080
6	4.242825	0.722800	-0.776155
6	2.973172	0.199003	-1.006430
6	-1.345986	2.743143	-0.441114
6	-1.272476	4.157508	0.128248
6	-2.464268	5.014820	-0.276159
6	3.775146	0.842197	1.599365
5	1.291173	-0.004620	2.433796
6	4.639883	1.040622	0.524561
8	1.504285	-0.930438	3.495250
8	0.660575	1.185848	2.923433
1	1.141883	-2.589525	-0.363613
1	0.816869	-1.649080	-1.827874
1	-0.523175	-2.116715	-0.744424
1	-1.439631	-0.706027	1.336403
1	-0.107881	0.562535	-1.587732
1	-0.119093	1.784495	1.087438
1	0.791419	2.335598	-0.327853
1	4.926036	0.877823	-1.606654
1	2.669000	-0.058426	-2.020394
1	-2.271945	2.259433	-0.102599
1	-1.397284	2.796159	-1.539320
1	-0.342150	4.635045	-0.205555
1	-1.208594	4.097982	1.221483
1	-2.390317	6.021876	0.144378
1	-3.404394	4.576141	0.075209
1	-2.532035	5.113904	-1.365274
1	4.098517	1.096928	2.606054
1	5.634676	1.445372	0.694140
1	1.882412	-1.743643	3.155866
1	0.236561	0.959753	3.756487
8	-2.284982	-0.531330	0.854969
1	-1.193200	0.152469	-0.369010
1	-2.745154	-1.373768	0.815284

TS_{Q-R}

6	0.546786	-1.764494	-0.830572
6	0.671809	-0.521401	0.041703
8	0.320569	-0.820848	1.361072
6	2.069600	0.053313	0.099756
7	-0.355957	0.477016	-0.473029
6	-0.197890	1.878885	-0.025348
6	2.476696	0.314157	1.408551

6	4.154245	0.839477	-0.785218
6	2.885119	0.309412	-1.000795
6	-1.409244	2.700664	-0.437024
6	-1.284642	4.159148	-0.007089
6	-2.493678	4.995634	-0.404537
6	3.753925	0.844984	1.600972
5	1.353117	-0.031571	2.510751
6	4.587318	1.103104	0.514977
8	1.600360	-0.984883	3.521154
8	0.658374	1.103049	2.993330
1	1.202530	-2.539148	-0.430136
1	0.836039	-1.571362	-1.868681
1	-0.481620	-2.140690	-0.815657
1	-0.888180	-0.739991	1.421056
1	-0.388598	0.437272	-1.489472
1	-0.090190	1.856118	1.065282
1	0.725338	2.295945	-0.442444
1	4.808194	1.042598	-1.628746
1	2.551675	0.099881	-2.015766
1	-2.310354	2.260130	0.009135
1	-1.543285	2.655623	-1.528887
1	-0.375683	4.590885	-0.446064
1	-1.145852	4.200347	1.080369
1	-2.382067	6.035550	-0.083683
1	-3.411011	4.604682	0.048798
1	-2.636583	4.996812	-1.490918
1	4.100874	1.059152	2.609207
1	5.581009	1.513227	0.676224
1	2.097274	-1.727726	3.173850
1	0.226599	0.862489	3.818400
8	-1.997561	-0.445754	1.066672
1	-1.388894	0.031679	0.101399
1	-2.506812	-1.232772	0.849091
R			
6	0.544823	-1.745872	-0.829877
6	0.631953	-0.483216	0.026636
8	0.207347	-0.781529	1.333719
6	2.061174	0.027521	0.171555
7	-0.287777	0.514378	-0.557554
6	-0.207203	1.869044	0.001314
6	2.530053	0.362142	1.449930
6	4.168989	0.693386	-0.812665
6	2.875977	0.200611	-0.948780
6	-1.394160	2.705141	-0.454648
6	-1.349766	4.130357	0.086726
6	-2.542684	4.968796	-0.353587
6	3.837714	0.852160	1.564634
5	1.629039	0.209936	2.747318
6	4.654860	1.017021	0.451192
8	1.786819	-0.820426	3.646887
8	0.927295	1.307852	3.181724
1	1.170275	-2.525664	-0.391368
1	0.872007	-1.579386	-1.860610
1	-0.491572	-2.096651	-0.848935
1	-0.774126	-0.841675	1.338787
1	-0.135546	0.550533	-1.561609
1	-0.205696	1.778071	1.092210
1	0.735908	2.365512	-0.272321
1	4.796050	0.821376	-1.690601
1	2.506118	-0.049505	-1.940748
1	-2.321549	2.214203	-0.131292
1	-1.426711	2.736857	-1.554448
1	-0.418839	4.613886	-0.237669
1	-1.305914	4.095982	1.182839
1	-2.489727	5.984744	0.049530
1	-3.484622	4.524752	-0.013431
1	-2.591183	5.046998	-1.445519
1	4.221910	1.116380	2.547484
1	5.665179	1.399727	0.566227
1	2.256039	-1.558681	3.252720
1	0.507128	1.107434	4.024692
8	-2.433084	-0.557747	0.725367
1	-1.885531	-0.123835	0.022859
1	-2.883619	-1.292021	0.298994
S			
6	-0.408850	-3.405021	-2.478250
6	-0.444552	-2.026270	-1.809720
8	1.406787	-1.729515	2.497190
6	0.959841	-1.621046	-1.334671
7	-0.951590	-1.083203	-2.742144
6	-1.018557	0.333571	-2.418229
6	1.417911	-1.494395	-0.001646
6	3.199280	-1.140991	-2.179853
6	1.860689	-1.431006	-2.392989
6	-2.100381	0.779473	-1.430589
6	-2.255780	2.296417	-1.393252
6	-3.309472	2.763493	-0.397298
6	2.791131	-1.218548	0.176584
5	0.640020	-1.492261	1.385342
6	3.674598	-1.044916	-0.877237
8	-0.671102	-1.200120	1.602698
8	-1.390376	-2.169426	-0.690885
1	-0.035504	-4.162987	-1.781271
1	0.247737	-3.398402	-3.349041
1	-1.422633	-3.671711	-2.793642
1	0.861612	-1.633199	3.284652
1	-1.821998	-1.417074	-3.144990
1	-0.032009	0.648263	-2.059123

1	-1.177362	0.860970	-3.368202
1	3.863488	-0.994019	-3.026997
1	1.478646	-1.495382	-3.407388
1	-1.856320	0.422888	-0.422624
1	-3.053899	0.309218	-1.706191
1	-2.512948	2.658759	-2.397981
1	-1.288446	2.754416	-1.145636
1	-3.403338	3.853925	-0.391679
1	-3.058619	2.445844	0.620623
1	-4.292917	2.345848	-0.639509
1	3.159417	-1.131654	1.193491
1	4.721084	-0.828071	-0.682657
1	-1.151015	-1.193682	0.757313
1	-1.165613	-2.981486	-0.217843
8	-3.659615	-2.302307	-2.386117
1	-4.463106	-2.758980	-2.125384
1	-3.092936	-2.283423	-1.598548

$TS_{S\text{-}M''}$

6	-0.448297	-3.176849	-3.047232
6	-0.202634	-1.935468	-2.241594
8	0.668356	0.519540	1.373790
6	1.131894	-1.737351	-1.626347
7	-1.062647	-0.968292	-2.419302
6	-1.004367	0.427749	-2.008053
6	1.358778	-1.103843	-0.381732
6	3.524275	-2.035188	-1.955117
6	2.215863	-2.204574	-2.386483
6	-2.224894	0.832736	-1.189660
6	-2.119269	2.280169	-0.720523
6	-3.342462	2.724997	0.070774
6	2.692999	-0.950153	0.020152
5	0.266321	-0.613606	0.680820
6	3.764920	-1.401862	-0.742002
8	-0.894128	-1.196189	0.940932
8	-1.455352	-2.975859	-0.565809
1	0.006363	-4.043219	-2.570734
1	-0.012582	-3.046382	-4.046177
1	-1.521676	-3.331548	-3.154591
1	-0.005932	0.705271	2.036331
1	-1.989518	-1.291463	-2.737456
1	-0.080240	0.604473	-1.456314
1	-0.953834	1.029971	-2.925609
1	4.346084	-2.396255	-2.566313
1	2.039516	-2.689436	-3.341273
1	-2.301733	0.163884	-0.326622
1	-3.132111	0.691283	-1.791178
1	-1.977776	2.944934	-1.584224
1	-1.219408	2.383868	-0.100087
1	-3.244591	3.760422	0.411096
1	-3.488231	2.093684	0.953500
1	-4.252299	2.657434	-0.535582
1	2.880277	-0.453574	0.967394
1	4.782846	-1.261179	-0.389123
1	-1.166159	-2.060894	0.297595
1	-1.296625	-3.841626	-0.178825
8	-3.396539	-2.302205	-2.104707
1	-3.958056	-3.024351	-2.394966
1	-2.775831	-2.675058	-1.398442

M''

6	-0.162540	-3.411492	-2.102398
6	-0.048962	-1.962318	-1.642965
8	0.420892	-1.713250	1.999463
6	1.394434	-1.509794	-1.523997
7	-0.835730	-1.147712	-2.475397
6	-0.919242	0.283405	-2.250711
6	1.808199	-1.378192	-0.195562
6	3.572945	-0.902710	-2.291798
6	2.261921	-1.270921	-2.583816
6	-1.965018	0.739986	-1.229565
6	-2.086398	2.257915	-1.151788
6	-3.130916	2.723119	-0.145218
6	3.124635	-1.010536	0.084342
5	0.545609	-1.695081	0.647852
6	4.004287	-0.776178	-0.967968
8	-0.501683	-1.970112	-0.203906
8	-3.200381	-2.269074	0.250888
1	0.381945	-4.071524	-1.424192
1	0.249082	-3.514256	-3.108979
1	-1.216775	-3.702955	-2.123610
1	-0.464364	-1.970021	2.276214
1	-1.744460	-1.569036	-2.672853
1	0.072235	0.647746	-1.957436
1	-1.146428	0.760528	-3.213883
1	4.269766	-0.707899	-3.102537
1	1.918493	-1.358911	-3.610858
1	-1.706228	0.340514	-0.240861
1	-2.936490	0.306307	-1.507283
1	-2.334401	2.651940	-2.146519
1	-1.109269	2.686826	-0.891865
1	-3.199378	3.814725	-0.109756
1	-2.889396	2.374507	0.865283
1	-4.124254	2.337059	-0.400083
1	3.459146	-0.903984	1.112747
1	5.031795	-0.487918	-0.764599
1	-2.232236	-2.196905	0.125716
1	-3.484749	-1.364703	0.415625
8	-3.555992	-2.367881	-2.460079
1	-3.890377	-3.205797	-2.788821

1	-3.570416	-2.441425	-1.482523

Hypothetic Mechanism 3

Z-Isomer

T

6	-0.433283	-3.417174	-2.105133
6	-0.415253	-1.952373	-1.678346
8	0.429775	-3.628394	1.285193
6	1.002355	-1.527792	-1.315253
7	-0.969353	-1.154865	-2.729084
6	-1.075348	0.290816	-2.596908
6	1.458642	-1.683550	0.003154
6	3.174003	-0.706684	-1.998580
6	1.864416	-1.058033	-2.308077
6	-2.072199	0.855444	-1.572868
6	-2.374887	2.330983	-1.818427
6	-3.317618	2.930464	-0.783356
6	2.779533	-1.314872	0.293845
5	0.554145	-2.275516	1.156544
6	3.633427	-0.827772	-0.690435
8	0.072761	-1.485157	2.178155
8	-1.224221	-1.943130	-0.457523
1	-0.085285	-4.042244	-1.280252
1	0.215050	-3.558298	-2.971561
1	-1.455820	-3.704611	-2.370444
1	-0.096879	-3.842502	2.062236
1	-1.862781	-1.553827	-3.005329
1	-0.077930	0.699769	-2.399854
1	-1.365051	0.667187	-3.586463
1	3.833777	-0.336865	-2.778579
1	1.491086	-0.969400	-3.324445
1	-1.670602	0.766940	-0.549543
1	-3.001546	0.271674	-1.603300
1	-2.810221	2.440226	-2.820590
1	-1.434107	2.897861	-1.833759
1	-3.526027	3.984011	-0.993082
1	-2.890305	2.871641	0.223919
1	-4.274492	2.397829	-0.766513
1	3.152773	-1.424845	1.310212
1	4.654148	-0.552114	-0.440436
1	0.317881	-0.565083	2.055110
1	-1.335017	-1.021839	-0.192263
8	-3.645265	-2.309326	-1.845058
1	-4.129677	-3.128114	-1.710931
1	-2.939302	-2.316472	-1.175674

TS_{T-U}

6	-0.435373	-3.433460	-2.115772
6	-0.297662	-1.949619	-1.977758
8	0.339714	-3.635449	0.987740
6	1.008996	-1.439221	-1.489168
7	-1.112171	-1.200169	-2.695721
6	-1.147259	0.256359	-2.729100
6	1.307255	-1.616286	-0.129919
6	3.193288	-0.536032	-1.947619
6	1.934057	-0.914453	-2.398918
6	-2.032927	0.865637	-1.643559
6	-2.305132	2.345758	-1.892877
6	-3.141719	2.982273	-0.790742
6	2.582451	-1.225118	0.284880
5	0.117537	-2.223031	0.817239
6	3.515023	-0.690912	-0.600571
8	-0.153211	-1.466386	2.021716
8	-1.160549	-1.993910	-0.031928
1	-0.092770	-3.916862	-1.195991
1	0.213434	-3.746618	-2.942279
1	-1.467456	-3.715894	-2.333677
1	-0.301747	-3.947469	1.633848
1	-1.995014	-1.656023	-2.922107
1	-0.125136	0.630391	-2.646404
1	-1.522761	0.540456	-3.718770
1	3.919779	-0.127957	-2.644266
1	1.680877	-0.822626	-3.453238
1	-1.535172	0.753076	-0.671948
1	-2.977191	0.308862	-1.588474
1	-2.816092	2.461413	-2.858228
1	-1.351119	2.881606	-1.986164
1	-3.331367	4.040114	-0.995068
1	-2.635357	2.915606	0.177929
1	-4.111056	2.482168	-0.691946
1	2.858055	-1.339786	1.331761
1	4.498338	-0.394952	-0.244038
1	0.633265	-1.440695	2.570896
1	-1.430049	-1.102048	0.222318
8	-3.407763	-2.390094	-1.551396
1	-4.029902	-3.096044	-1.359451
1	-2.728809	-2.434611	-0.846929

U

6	-0.450320	-3.499244	-2.170726
6	-0.220525	-2.040272	-2.229913
8	0.168196	-3.573719	0.757901
6	1.039728	-1.499741	-1.687413
7	-1.139959	-1.268830	-2.720268
6	-1.134507	0.189477	-2.768852
6	1.179546	-1.522947	-0.284454
6	3.188434	-0.462189	-2.014315
6	2.014354	-0.979390	-2.546579
6	-2.100357	0.784580	-1.750333
6	-2.124749	2.307327	-1.821471

6	-3.081472	2.918571	-0.805793
6	2.363539	-0.954321	0.201601
5	0.056765	-2.108332	0.784258
6	3.355523	-0.445701	-0.631401
8	0.248778	-1.556350	2.117502
8	-1.326612	-1.666876	0.367449
1	-0.286802	-3.811075	-1.120442
1	0.327176	-3.990750	-2.765947
1	-1.443501	-3.784475	-2.519734
1	-0.543737	-3.916149	1.308554
1	-2.059730	-1.714253	-2.819391
1	-0.114115	0.530107	-2.585570
1	-1.412390	0.481015	-3.788864
1	3.958645	-0.069810	-2.671798
1	1.859391	-0.991831	-3.623584
1	-1.790856	0.440898	-0.757443
1	-3.106662	0.380343	-1.917607
1	-2.409290	2.626605	-2.833671
1	-1.112239	2.696244	-1.651110
1	-3.083960	4.010893	-0.866574
1	-2.799168	2.641350	0.215164
1	-4.107363	2.572208	-0.970962
1	2.484532	-0.905969	1.280379
1	4.263560	-0.028615	-0.203091
1	0.786490	-2.176647	2.614101
1	-1.665968	-1.225518	1.152929
8	-3.269229	-2.277171	-1.399580
1	-3.742991	-3.074582	-1.152274
1	-2.604851	-2.122867	-0.678378

V

6	-0.463943	-3.351891	-2.600847
6	-0.247080	-1.962989	-2.120693
8	-0.655018	-2.978002	0.387196
6	1.101071	-1.550368	-1.692938
7	-1.249337	-1.157850	-2.017389
6	-1.212227	0.229633	-1.553757
6	1.342518	-1.504800	-0.302711
6	3.357467	-0.910336	-2.242410
6	2.084962	-1.287740	-2.651869
6	-2.481380	0.951037	-1.977824
6	-2.492795	2.398581	-1.497510
6	-3.763231	3.139527	-1.892605
6	2.646650	-1.132210	0.055182
5	0.240589	-1.867445	0.891972
6	3.633037	-0.830549	-0.879377
8	0.925584	-2.294843	2.102056
8	-0.598059	-0.719985	1.228888
1	-0.230167	-4.006332	-1.755103
1	0.241843	-3.569517	-3.408554
1	-1.488434	-3.528934	-2.932449
1	-0.839130	-3.458393	1.202095
1	-2.179823	-1.605126	-2.025572
1	-1.100052	0.201877	-0.457608
1	-0.317040	0.697177	-1.975086
1	4.124097	-0.686531	-2.978274
1	1.851206	-1.359393	-3.712220
1	-3.347592	0.421127	-1.558839
1	-2.588017	0.923732	-3.071321
1	-1.618043	2.923469	-1.903841
1	-2.378262	2.413993	-0.406460
1	-3.748502	4.173444	-1.535403
1	-4.650108	2.654548	-1.471006
1	-3.886206	3.165549	-2.980932
1	2.880749	-1.088666	1.116666
1	4.626595	-0.541190	-0.545917
1	1.614815	-2.925540	1.880787
1	-0.187225	-0.284383	1.979858
8	-3.080736	-2.601662	-0.778660
1	-3.489480	-3.469994	-0.795126
1	-2.259998	-2.701021	-0.226100

TS_{V-W}

6	-0.495013	-3.343744	-2.599848
6	-0.268847	-1.958032	-2.120419
8	-0.394295	-3.262344	0.346885
6	1.084460	-1.539366	-1.712960
7	-1.268140	-1.152238	-1.984202
6	-1.213585	0.242513	-1.545897
6	1.340989	-1.532138	-0.323886
6	3.314974	-0.822128	-2.264104
6	2.047835	-1.219188	-2.673685
6	-2.465470	0.976974	-1.999061
6	-2.452568	2.436897	-1.557681
6	-3.706940	3.190272	-1.979777
6	2.635286	-1.131927	0.033738
5	0.269001	-2.014510	0.850402
6	3.602332	-0.774605	-0.902223
8	0.932696	-2.246372	2.122632
8	-0.773801	-1.001092	1.084753
1	-0.255509	-3.995571	-1.751769
1	0.203958	-3.560343	-3.413772
1	-1.521405	-3.515232	-2.931464
1	-0.634266	-3.734364	1.151251
1	-2.198462	-1.592126	-1.935499
1	-1.118459	0.223861	-0.450320
1	-0.305981	0.687218	-1.963820
1	4.067584	-0.554729	-2.999943
1	1.803008	-1.260596	-3.733064
1	-3.346341	0.476206	-1.574584

1	-2.563162	0.922658	-3.092229
1	-1.566011	2.934206	-1.972777
1	-2.343298	2.480510	-0.466746
1	-3.674806	4.232897	-1.650160
1	-4.605148	2.733265	-1.550924
1	-3.823318	3.189572	-3.069098
1	2.874160	-1.105965	1.094432
1	4.590241	-0.464722	-0.570384
1	1.627144	-2.899765	2.011805
1	-0.709004	-0.747635	2.009347
8	-3.060033	-2.466066	-0.482463
1	-3.223965	-3.411136	-0.544375
1	-2.248541	-2.413117	0.066190

W

6	-0.507649	-3.350263	-2.685845
6	-0.292343	-1.979730	-2.159760
8	-0.398686	-3.368604	0.269238
6	1.053420	-1.553511	-1.737592
7	-1.298310	-1.190825	-1.975837
6	-1.236245	0.201560	-1.532335
6	1.279738	-1.557950	-0.341567
6	3.281935	-0.792811	-2.233454
6	2.030880	-1.208234	-2.673867
6	-2.466784	0.954204	-2.015938
6	-2.444101	2.414550	-1.576002
6	-3.675767	3.185439	-2.032067
6	2.558649	-1.137301	0.046422
5	0.209178	-2.106347	0.809941
6	3.538244	-0.751643	-0.865573
8	0.872604	-2.352508	2.077363
8	-0.868896	-1.126573	1.094904
1	-0.226793	-4.041605	-1.884204
1	0.166993	-3.513560	-3.532956
1	-1.540426	-3.527597	-2.993941
1	-0.584231	-3.891183	1.056942
1	-2.223048	-1.638693	-1.852161
1	-1.163165	0.186488	-0.436465
1	-0.315154	0.636947	-1.928689
1	4.045456	-0.505192	-2.950071
1	1.808109	-1.242327	-3.738197
1	-3.368231	0.468012	-1.618171
1	-2.536065	0.898423	-3.110757
1	-1.539768	2.897706	-1.968691
1	-2.362344	2.459771	-0.482541
1	-3.636861	4.227925	-1.702947
1	-4.591952	2.742608	-1.627021
1	-3.762945	3.184641	-3.124010
1	2.778268	-1.117603	1.111406
1	4.512231	-0.425641	-0.509184
1	1.618843	-2.942595	1.949901
1	-0.737749	-0.853859	2.007472
8	-2.951101	-2.413643	-0.288914
1	-2.190611	-3.024054	-0.182670
1	-2.640024	-1.746426	0.350140

$TS_{W\text{-}N'}$

6	-0.505049	-3.305858	-2.602514
6	-0.333952	-1.914830	-2.082671
8	-0.686082	-3.044778	0.444391
6	1.035018	-1.495712	-1.704763
7	-1.375890	-1.192486	-1.870647
6	-1.310295	0.199743	-1.439017
6	1.336308	-1.508469	-0.324825
6	3.274950	-0.825482	-2.300948
6	1.986407	-1.183602	-2.678986
6	-2.494136	0.971828	-2.006159
6	-2.481977	2.435287	-1.577931
6	-3.665588	3.222169	-2.125425
6	2.651370	-1.151951	0.006736
5	0.323407	-1.881382	0.915430
6	3.604214	-0.809496	-0.948834
8	1.005060	-2.466515	2.047835
8	-0.446899	-0.743404	1.318736
1	-0.171258	-4.011895	-1.836845
1	0.137627	-3.440308	-3.479269
1	-1.539425	-3.514146	-2.884583
1	-0.703335	-3.595396	1.236049
1	-2.321775	-1.850758	-1.373502
1	-1.312940	0.212849	-0.341697
1	-0.360511	0.640098	-1.761558
1	4.012772	-0.564820	-3.054184
1	1.712334	-1.203729	-3.731610
1	-3.426020	0.497786	-1.669717
1	-2.489304	0.905672	-3.102676
1	-1.544773	2.902365	-1.908516
1	-2.476016	2.489632	-0.481722
1	-3.634771	4.267556	-1.803596
1	-4.615386	2.796814	-1.783205
1	-3.675856	3.211955	-3.220977
1	2.924198	-1.141632	1.059533
1	4.608576	-0.532728	-0.638663
1	1.727866	-3.026837	1.757327
1	-0.619992	-0.818790	2.261148
8	-2.853850	-2.506073	-0.477963
1	-3.263724	-3.305318	-0.823269
1	-1.850003	-2.781360	0.059386

N'(3)

6	-0.546407	-3.179416	-2.361125

6	-0.471318	-1.766087	-1.862692
8	-0.782459	-4.624005	0.525993
6	0.916479	-1.263650	-1.582091
7	-1.553319	-1.115632	-1.675332
6	-1.535184	0.260697	-1.220139
6	1.458705	-1.338297	-0.282121
6	3.017215	-0.407485	-2.435106
6	1.702550	-0.812919	-2.644187
6	-2.341058	1.136612	-2.174256
6	-2.465522	2.576198	-1.687562
6	-3.258268	3.459471	-2.642391
6	2.793811	-0.949097	-0.104818
5	0.645414	-1.833424	0.971863
6	3.568075	-0.477452	-1.160039
8	1.175082	-2.730331	1.870945
8	-0.586587	-1.324631	1.216872
1	-0.302210	-3.875057	-1.546735
1	0.172649	-3.340052	-3.171290
1	-1.555190	-3.395873	-2.719273
1	-0.270490	-4.287269	1.268084
1	-2.592231	-2.242156	-0.556061
1	-1.996484	0.279148	-0.225564
1	-0.517829	0.660564	-1.105137
1	3.610977	-0.044879	-3.269328
1	1.277355	-0.776864	-3.644128
1	-3.337881	0.696026	-2.304150
1	-1.867600	1.120136	-3.165348
1	-1.462814	2.999805	-1.541676
1	-2.942331	2.582468	-0.698211
1	-3.337630	4.485253	-2.269328
1	-4.275111	3.076653	-2.782570
1	-2.783932	3.500433	-3.629126
1	3.232346	-0.996399	0.890543
1	4.595041	-0.168323	-0.988648
1	2.009886	-3.093983	1.562416
1	-1.040852	-1.754463	1.948808
8	-2.923777	-2.834777	0.151864
1	-3.665036	-3.303016	-0.242266
1	-1.558766	-4.033930	0.469611

***E*-Isomer**

T’

6	0.544823	-1.745872	-0.829877
6	0.631953	-0.483216	0.026636
8	0.207347	-0.781529	1.333719
6	2.061174	0.027521	0.171555
7	-0.287777	0.514378	-0.557554
6	-0.207203	1.869044	0.001314
6	2.530053	0.362142	1.449930
6	4.168989	0.693386	-0.812665
6	2.875977	0.200611	-0.948780
6	-1.394160	2.705141	-0.454648
6	-1.349766	4.130357	0.086726
6	-2.542684	4.968796	-0.353587
6	3.837714	0.852160	1.564634
5	1.629039	0.209936	2.747318
6	4.654860	1.017021	0.451192
8	1.786819	-0.820426	3.646887
8	0.927295	1.307852	3.181724
1	1.170275	-2.525664	-0.391368
1	0.872007	-1.579386	-1.860610
1	-0.491572	-2.096651	-0.848935
1	-0.774126	-0.841675	1.338787
1	-0.135546	0.550533	-1.561609
1	-0.205696	1.778071	1.092210
1	0.735908	2.365512	-0.272321
1	4.796050	0.821376	-1.690601
1	2.506118	-0.049505	-1.940748
1	-2.321549	2.214203	-0.131292
1	-1.426711	2.736857	-1.554448
1	-0.418839	4.613886	-0.237669
1	-1.305914	4.095982	1.182839
1	-2.489727	5.984744	0.049530
1	-3.484622	4.524752	-0.013431
1	-2.591183	5.046998	-1.445519
1	4.221910	1.116380	2.547484
1	5.665179	1.399727	0.566227
1	2.256039	-1.558681	3.252720
1	0.507128	1.107434	4.024692
8	-2.433084	-0.557747	0.725367
1	-1.885531	-0.123835	0.022859
1	-2.883619	-1.292021	0.298994

$TS_{T'-U'}$

6	0.592728	-2.355065	-1.461956
6	0.007194	-1.019297	-1.142911
8	-0.625287	-1.756059	0.899845
6	0.858855	0.009692	-0.478900
7	-1.084830	-0.598943	-1.715207
6	0.616569	0.333306	0.863023
6	2.662530	1.597563	-0.625988
6	1.868572	0.624961	-1.225442
6	1.437457	1.309975	1.433980
5	-0.576619	-0.460614	1.645003
6	2.445537	1.939382	0.707813
8	-0.371641	-0.588124	3.068053
8	-1.895358	0.211740	1.482646
6	-5.136958	-0.547657	-4.614441
6	-4.122105	-1.401609	-3.865573
6	-3.055993	-0.566615	-3.165061

6	-2.038451	-1.429973	-2.433704
1	1.273269	-2.645420	-0.664205
1	1.158791	-2.276817	-2.399672
1	-0.169634	-3.126467	-1.577487
1	-1.529785	-2.078818	0.976430
1	-1.425763	0.353246	-1.419809
1	3.447973	2.083253	-1.198009
1	2.032825	0.355544	-2.266836
1	1.279741	1.561462	2.480790
1	3.070265	2.694022	1.179222
1	0.403897	-1.127440	3.234239
1	-2.174891	0.475784	2.363804
1	-5.896267	-1.166259	-5.101610
1	-4.651345	0.055823	-5.388674
1	-5.651459	0.139509	-3.934693
1	-3.640939	-2.099568	-4.564079
1	-4.640979	-2.022962	-3.123569
1	-3.524798	0.121717	-2.451082
1	-2.532204	0.060504	-3.898051
1	-2.546120	-2.088190	-1.713810
1	-1.505570	-2.071405	-3.144113
8	-2.039343	1.687907	-0.639436
1	-1.320774	2.322092	-0.550117
1	-2.063593	1.221434	0.249343

U’

6	0.756431	-2.242357	-1.729119
6	0.160002	-0.920299	-1.410876
8	-0.700939	-1.829646	0.928791
6	0.951148	0.039318	-0.608794
7	-1.022894	-0.560489	-1.779759
6	0.547279	0.312008	0.712972
6	2.827296	1.546791	-0.464914
6	2.087122	0.619559	-1.186691
6	1.337320	1.236060	1.409862
5	-0.690093	-0.466957	1.482821
6	2.444575	1.856812	0.839269
8	-0.535749	-0.416788	2.928778
8	-2.004128	0.210724	1.227291
6	-5.246885	-0.605496	-4.429695
6	-4.221324	-1.424388	-3.656786
6	-3.067885	-0.575188	-3.133974
6	-2.053288	-1.405249	-2.361108
1	0.610416	-2.835415	-0.817966
1	1.830969	-2.133594	-1.889934
1	0.297029	-2.754522	-2.575805
1	-1.477833	-2.264741	1.293351
1	-1.359632	0.394328	-1.451508
1	3.697344	2.017403	-0.913485
1	2.376687	0.367083	-2.205006
1	1.066329	1.439886	2.443254
1	3.025830	2.572241	1.415929
1	0.146853	-1.040084	3.186211
1	-2.329719	0.472842	2.093672
1	-6.067081	-1.232396	-4.791478
1	-4.791372	-0.118519	-5.298611
1	-5.678590	0.179397	-3.800027
1	-3.827972	-2.223740	-4.299465
1	-4.712077	-1.926227	-2.812403
1	-3.449970	0.221440	-2.484284
1	-2.563371	-0.075883	-3.971424
1	-2.532982	-1.929983	-1.523896
1	-1.600545	-2.162473	-3.007824
8	-1.989446	1.728940	-0.805007
1	-1.273273	2.357646	-0.669314
1	-2.072087	1.245568	0.082705

$TS_{U'-V'}$

6	0.667927	-2.270803	-1.640800
6	0.091837	-0.915025	-1.404148
8	-0.828780	-1.758451	1.044908
6	0.910699	0.030588	-0.590786
7	-1.053440	-0.518034	-1.823868
6	0.587573	0.284319	0.756727
6	2.806539	1.530332	-0.509886
6	2.013025	0.629349	-1.210795
6	1.419284	1.189201	1.430563
5	-0.649012	-0.423763	1.567694
6	2.504696	1.811092	0.819824
8	-0.517204	-0.323291	3.008175
8	-1.956579	0.391041	1.338419
6	-5.294076	-0.665361	-4.454838
6	-4.138765	-1.458162	-3.857661
6	-3.131685	-0.576986	-3.127874
6	-1.982193	-1.380998	-2.536968
1	0.493936	-2.819303	-0.706885
1	1.747659	-2.203238	-1.792225
1	0.206652	-2.813693	-2.467266
1	-1.553756	-2.163785	1.529997
1	-1.501238	0.607961	-1.348508
1	3.653874	2.003823	-0.997723
1	2.242515	0.400878	-2.249919
1	1.197084	1.388305	2.476651
1	3.122452	2.507392	1.381484
1	0.203656	-0.885845	3.298363
1	-2.103968	0.892724	2.147213
1	-6.003767	-1.319179	-4.970468
1	-4.934569	0.073199	-5.179474
1	-5.843657	-0.123562	-3.677768
1	-3.626590	-2.017306	-4.652635

1	-4.531700	-2.212218	-3.162364
1	-3.632708	-0.017576	-2.328459
1	-2.725712	0.174229	-3.818212
1	-2.365225	-2.127506	-1.827150
1	-1.467584	-1.930617	-3.335950
8	-1.929061	1.540364	-0.757057
1	-1.226849	2.200212	-0.738199
1	-1.975784	1.051302	0.321620

V'

6	0.600263	-2.322159	-1.576527
6	0.035744	-0.946451	-1.395032
8	-0.838430	-1.794379	1.134841
6	0.869338	0.002396	-0.589005
7	-1.079401	-0.518640	-1.850223
6	0.609417	0.233498	0.776991
6	2.726079	1.559653	-0.568204
6	1.920881	0.649531	-1.245416
6	1.443440	1.153930	1.427043
5	-0.569112	-0.488549	1.631510
6	2.483850	1.813296	0.778018
8	-0.453796	-0.348017	3.064186
8	-1.933974	0.400668	1.453339
6	-5.278461	-0.678130	-4.541188
6	-4.117033	-1.467909	-3.951641
6	-3.133247	-0.593867	-3.182999
6	-1.973109	-1.387294	-2.598955
1	0.453405	-2.839737	-0.621675
1	1.676004	-2.269351	-1.764999
1	0.115774	-2.893415	-2.370172
1	-1.480290	-2.213185	1.715813
1	-1.601132	0.910536	-1.251882
1	3.534931	2.064068	-1.089535
1	2.102355	0.447007	-2.299014
1	1.261002	1.343956	2.482590
1	3.107258	2.518996	1.321055
1	0.312643	-0.833630	3.375301
1	-1.980562	0.946645	2.247541
1	-5.971137	-1.327936	-5.084647
1	-4.922268	0.086989	-5.239499
1	-5.846798	-0.167484	-3.756351
1	-3.585571	-1.995159	-4.755794
1	-4.505736	-2.249299	-3.284481
1	-3.653885	-0.069823	-2.371190
1	-2.733610	0.187414	-3.843109
1	-2.356188	-2.167412	-1.925097
1	-1.439729	-1.904356	-3.410090
8	-1.887604	1.707278	-0.682656
1	-1.114417	2.282383	-0.676601
1	-1.941389	0.999547	0.605507

W'

6	0.362026	-2.398018	-1.345925
6	-0.110995	-0.977583	-1.299637
8	-0.241930	-2.093791	1.675447
6	0.771161	-0.017518	-0.555747
7	-1.162520	-0.501475	-1.848808
6	0.754853	0.101608	0.850212
6	2.522103	1.653126	-0.740557
6	1.637320	0.757407	-1.333073
6	1.668659	1.003933	1.410894
5	-0.234631	-0.701990	1.838808
6	2.543577	1.769094	0.644017
8	-0.299139	-0.294238	3.196453
8	-1.870470	-0.182359	1.280043
6	-5.159655	-0.495641	-4.829737
6	-4.113683	-1.339510	-4.112584
6	-3.116260	-0.501961	-3.320855
6	-2.069330	-1.347456	-2.609712
1	0.301352	-2.792338	-0.324402
1	1.414520	-2.428316	-1.645238
1	-0.225598	-3.027236	-2.015824
1	-0.605586	-2.503050	2.465553
1	-1.659396	0.995778	-1.178807
1	3.190344	2.247642	-1.357075
1	1.614415	0.653458	-2.415239
1	1.714690	1.096835	2.494314
1	3.238245	2.451265	1.126580
1	-0.122902	0.643337	3.297541
1	-2.304867	0.062515	2.104672
1	-5.861479	-1.118437	-5.392690
1	-4.690290	0.198724	-5.535027
1	-5.740399	0.101274	-4.118302
1	-3.572904	-1.954560	-4.844923
1	-4.612552	-2.045983	-3.435425
1	-3.646218	0.107966	-2.577717
1	-2.607841	0.204786	-3.989824
1	-2.565763	-2.054563	-1.929689
1	-1.522395	-1.951788	-3.348109
8	-1.902654	1.693170	-0.498744
1	-1.103783	2.225286	-0.417439
1	-1.902521	0.614208	0.658331

$TS_{W'-N''}$

6	0.437900	-2.366138	-1.512557
6	-0.122564	-0.993933	-1.272130
8	-0.033295	-2.197492	1.568932
6	0.801058	-0.023457	-0.591540
7	-1.266555	-0.537233	-1.597265
6	0.927672	0.042190	0.810991
6	2.383610	1.797367	-0.856808

6	1.520249	0.854267	-1.405980
6	1.804924	1.004863	1.334276
5	0.150525	-0.875735	1.836102
6	2.532149	1.870320	0.523626
8	-0.154511	-0.414299	3.097847
8	-2.478398	-0.383662	1.354311
6	-4.834972	-0.493348	-5.068309
6	-4.157430	-1.281589	-3.954753
6	-2.982769	-0.535483	-3.332139
6	-2.299810	-1.330841	-2.224847
1	0.517008	-2.876513	-0.547715
1	1.448468	-2.281193	-1.924494
1	-0.171600	-2.970979	-2.184990
1	-0.584571	-2.606175	2.243757
1	-1.748142	1.038889	-0.903057
1	2.934846	2.471542	-1.506100
1	1.390482	0.801234	-2.483833
1	1.942676	1.064667	2.412846
1	3.208508	2.595758	0.966394
1	-0.009689	0.531932	3.174545
1	-2.430220	-0.148149	2.284634
1	-5.676190	-1.046306	-5.497314
1	-4.132804	-0.272989	-5.879863
1	-5.219122	0.462773	-4.696934
1	-3.811375	-2.247566	-4.347182
1	-4.890575	-1.517810	-3.172108
1	-3.322347	0.422879	-2.919361
1	-2.243789	-0.293238	-4.107837
1	-3.029569	-1.561694	-1.436591
1	-1.941710	-2.291145	-2.621302
8	-2.016094	1.772826	-0.293116
1	-1.177873	2.180415	-0.054581
1	-2.408598	0.466648	0.872202

N”

6	0.752756	-2.063695	-2.183818
6	0.209126	-0.847248	-1.466642
8	-0.694467	-1.272068	1.484831
6	1.207135	-0.001379	-0.747252
7	-1.024933	-0.520963	-1.418633
6	1.008254	0.403258	0.587540
6	3.307908	1.214683	-0.825180
6	2.358410	0.401539	-1.432957
6	1.985418	1.215493	1.179114
5	-0.262338	-0.005363	1.430750
6	3.126043	1.619274	0.492963
8	-0.912169	0.995988	2.149481
8	-3.342423	-1.276268	1.770305
6	-4.838882	-0.485191	-4.633757
6	-4.007439	-1.308479	-3.658610
6	-2.875122	-0.506926	-3.025593
6	-2.039568	-1.338914	-2.055481
1	0.407689	-2.969175	-1.673017
1	1.842597	-2.076742	-2.169503
1	0.414666	-2.118700	-3.222858
1	-1.625818	-1.365016	1.801670
1	-2.208728	0.547991	-0.442480
1	4.187330	1.528740	-1.379926
1	2.503104	0.095510	-2.466032
1	1.863109	1.520463	2.217567
1	3.869204	2.241134	0.983767
1	-0.464658	1.838361	2.023515
1	-3.696260	-0.892615	2.577720
1	-5.643613	-1.080145	-5.076352
1	-4.221295	-0.099185	-5.452257
1	-5.296652	0.374614	-4.133028
1	-3.592252	-2.183393	-4.177341
1	-4.655537	-1.704962	-2.865961
1	-3.285637	0.359017	-2.492069
1	-2.218325	-0.107526	-3.810244
1	-2.688384	-1.715686	-1.251990
1	-1.636904	-2.218840	-2.573519
8	-2.952021	0.861838	0.122797
1	-2.489553	1.292220	0.854515
1	-3.366267	-0.542041	1.111724

X

6	2.250005	-2.144490	-1.764721
6	2.018326	-0.796795	-1.125263
8	-0.171559	-0.634978	1.085959
6	2.854085	-0.487330	0.073067
7	1.164337	0.049972	-1.561840
6	2.308009	-0.046600	1.294636
6	5.092406	-0.398055	1.018760
6	4.238599	-0.662205	-0.045092
6	3.191979	0.199598	2.355688
5	0.768132	0.214214	1.520730
6	4.565885	0.025697	2.234213
8	0.406735	1.375621	2.199152
6	0.387683	-0.319977	-2.739200
6	-0.740732	0.655326	-3.041450
6	-1.461100	0.308137	-4.339785
6	-2.615854	1.253935	-4.643933
1	3.079165	-2.678665	-1.301367
1	2.433101	-2.058115	-2.841093
1	1.348605	-2.756832	-1.641040
1	0.413643	1.642525	-0.856061
1	6.164004	-0.528508	0.898882
1	4.657147	-0.991974	-0.992666
1	2.788884	0.511525	3.318460

1	5.219731	0.215749	3.080447
1	1.189176	1.864747	2.471596
1	1.060690	-0.372154	-3.610625
1	-0.034858	-1.329339	-2.621931
1	-0.339841	1.674866	-3.099339
1	-1.457439	0.649358	-2.212040
1	-1.837801	-0.722454	-4.283680
1	-0.743532	0.323120	-5.171972
1	-3.118454	0.987507	-5.578882
1	-2.265205	2.287569	-4.735828
1	-3.362748	1.230552	-3.843481
8	-0.163626	2.347069	-0.479783
1	0.137693	2.426493	0.434233
1	-1.614419	1.462333	-0.018977
8	-2.271062	0.849216	0.391155
1	-2.695530	1.368779	1.079745
1	-1.064461	-0.217510	1.015129

$TS_{X\text{-}O}$

6	2.419365	-2.166576	-1.900507
6	2.138719	-0.874744	-1.170173
8	-0.146517	-0.518936	1.110974
6	2.950424	-0.593333	0.046183
7	1.243728	-0.035307	-1.536533
6	2.371517	-0.032776	1.200953
6	5.127288	-0.613451	1.127480
6	4.320405	-0.880759	0.027571
6	3.205396	0.222863	2.298034
5	0.846210	0.351872	1.321866
6	4.566308	-0.066116	2.275807
8	0.568390	1.646544	1.777525
6	0.463411	-0.373089	-2.716622
6	-0.644652	0.629476	-2.994943
6	-1.422852	0.305956	-4.265823
6	-2.550045	1.293712	-4.538092
1	3.197446	-2.755400	-1.415681
1	2.707249	-1.984375	-2.942419
1	1.508313	-2.775421	-1.927544
1	-0.267452	2.145013	-0.995385
1	6.190102	-0.833682	1.085842
1	4.770616	-1.297015	-0.869692
1	2.772147	0.638762	3.206457
1	5.182926	0.132859	3.147691
1	1.390259	2.132326	1.899110
1	1.123888	-0.433243	-3.597320
1	0.014744	-1.373535	-2.610624
1	-0.210071	1.635389	-3.083483
1	-1.334971	0.644807	-2.142634
1	-1.835738	-0.708819	-4.187355
1	-0.732890	0.287395	-5.120559
1	-3.093771	1.039697	-5.453136
1	-2.164277	2.312649	-4.651370
1	-3.270248	1.304968	-3.713252
8	-0.818968	2.646349	-0.380160
1	-0.335902	2.543365	0.458830
1	-2.031305	1.487485	0.159690
8	-2.469419	0.727847	0.608236
1	-2.943366	1.113808	1.350108
1	-1.048608	-0.118637	1.068754

Wiberg Index Structure

O (**89**)

6	1.788312	0.514803	-0.783557
6	2.432810	-0.720470	-0.677200
6	1.806341	-1.803646	-0.060360
6	0.522710	-1.660612	0.459243
6	-0.101429	-0.419958	0.344320
6	0.507757	0.686423	-0.269331
6	-1.443514	-0.065875	0.833155
6	-2.306098	-1.043849	1.556955
5	-0.495477	1.967755	-0.257321
8	-0.981883	2.408860	-1.535225
8	-0.155446	3.067252	0.580455
6	-6.222725	2.727751	-1.001697
6	-5.199426	2.703520	0.126490
6	-3.967045	1.873539	-0.214141
6	-2.950206	1.856904	0.926258
7	-1.740730	1.156959	0.560852
1	2.299610	1.345515	-1.264785
1	3.436843	-0.842669	-1.075814
1	2.320370	-2.757354	0.015517
1	0.030691	-2.502275	0.940983
1	-3.275778	-0.633360	1.837811
1	-1.792847	-1.391313	2.459586
1	-2.469902	-1.922669	0.924908
1	-0.854769	1.733130	-2.203813
1	-0.229089	3.860515	0.043634
1	-7.094752	3.334301	-0.739095
1	-5.789075	3.144957	-1.916639
1	-6.578309	1.718306	-1.236033
1	-5.667583	2.310531	1.039572
1	-4.888189	3.729471	0.362752
1	-3.467131	2.269229	-1.104926
1	-4.266414	0.842026	-0.447420
1	-3.394434	1.434355	1.835536
1	-2.630656	2.878567	1.160879

VI.2.3. Experimental Details of Reversibility

VI.2.3.1. Preliminary ^{1}H-NMR reversibility study

2-Acetylbenzeneboronic acid (**88**, 8,0 mg, 0.05 mmol) was added to an eppendorf tube and then dissolved in 0.3 mL of D_2O. Afterwards, 1 equivalent of 1-butylamine (**87**, 5 µL) was added to the same tube and these compounds reacted for 18 h at 25 ºC. Subsequently, a ^{1}H-NMR spectrum was collected to evaluate the conjugation rate. Then, 0.1 mL of D_2O containing 1 eq of different molecules (described in Table **20** and Table **21**) were added to the tube. From that moment on, several ^{1}H-NMR spectra were collected in order to study the reaction's reversibility. Results are shown in Figure **26** (*p.72*) and resumed in Table **20** and Table **21**.

Table **20** - Reversibility Study, imine conversions (%) from ^{1}H-NMR in D_2O (1)

t (min)	D-Glucose	D-Fructose	D-Glutathione Reduced	D-Lactose	D-Sucrose
0	94	87	81	87	89
45	82	44	16	84	87
120	81	41	11	84	87
1440	81	44	7	84	87

Table **21** - Reversibility Study, imine conversions (%) from 1H-NMR in D_2O (2)

t (min)	Adenine	Cytosine	Thymine	Ephedrine	Dopamine Hydrochloride
0	82	93	90	86	86
45	82	91	85	84	50
120	82	91	86	84	24
1440	81	91	86	84	21

VI.2.3.2. Reversibility study with Lysozyme

To study reversibility of the link formed, equal quantities of reduced glutathione, dopamine hydrochloride and D-fructose (10.0 mM) were added to the modified lysozyme with 2-formylbenzeneboronic acid and the results were evaluated through ESI-FTICR-MS, detecting absence of conjugates or the decrease in their number, thus proving its reversibility. Results are shown in Figure **27** (*p.73*).

VI.3. Experimental Section of Chapter III

VI.3.1. Experimental Details of strategies to achiveve a modifiable backbone

VI.3.1.1. Modification of 2-carbonylbenzeneboronic acid

VI.3.1.1.1. *Nitration of 2-acetylbenzeneboronic acids*

- Synthesis of 2-acetyl-X-nitrobenzeneboronic acid

Literature basis - Keana, J. F. W.; Cai, X. S. *Bioconjugate Chemistry* **1991**, *2*, 317.

- General nitration procedure

A solution of fuming nitric acid was prepared at -50 ºC by adding dropwise 0.63 mL of sulphuric acid (12.2 mmol, 4 eq) into 0.51 mL of nitric acid (12.2 mmol, 4 eq). Seperately, 2-acetylbenzeneboronic acid (**88**, 0.5 g, 3.0 mmol) was added to a 25 mL round bottom flask and then dissolved in 1 mL of sulphuric acid (18.3 mmol, 6 eq) at -98 ºC. Then the solution of fuming nitric acid was slowly added to the solution containing compound **88** and left reacting for 40 min at -98 ºC (Scheme **36**, *p.81*). After that time, the reaction mixture was poured into ice-water and left for 10 min. Since no crystals were detected (as it had happened in the reported work from literature basis), a multiple extraction using ethyl acetate was performed. Several recrystallization attemps and column cromatographies never afforded the expected product.

Species with three aromatic proton signals, but without alkylic proton signals:

^{1}H NMR (400 MHz, D_2O) δ 8.82 (d, *J* = 1.9 Hz, 1H, C$\underline{H}_{arom}$), 8.46 (dd, *J* = 8.1, 2.1 Hz, 1H, C$\underline{H}_{arom}$), 7.72 (d, *J* = 8.1 Hz, 1H, C$\underline{H}_{arom}$).

^{1}H NMR (400 MHz, D_2O) δ 8.23 (d, *J* = 7.0 Hz, 2H, 2xC$\underline{H}_{arom}$), 7.62 (d, *J* = 8.2 Hz, 1H, C$\underline{H}_{arom}$).

Species bearing four aromatic proton signals:

^{1}H NMR (400 MHz, D_2O) δ 8.09 (d, *J* = 7.7 Hz, 1H, C$\underline{H}_{arom}$), 7.70 (t, *J* = 7.3 Hz, 1H, C$\underline{H}_{arom}$), 7.57 (t, *J* = 9.0 Hz, 2H, 2xC$\underline{H}_{arom}$), 2.70 (s, 3H, $C_{arom}COC\underline{H}_3$).

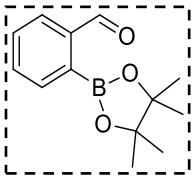
VI.3.1.1.2. *Methylation of 2-formylbenzeneboronic acids*

- Synthesis of 2-formylbenzeneboronic acid derivatives

Step 1 - Pinacol protection of 2-formylbenzeneboronic acid – Synthesis of compound **109** (R = H)

Literature basis - Lautens, M.; Mancuso, J. *Journal of Organic Chemistry* **2004**, *69 (10)*, 3478 and Vogels, C.M.; Nikolcheva, L.G.; Norman, D.W.; Spinney, H.A.; Decken, A.; Baerlocher, M.O.; Westcott, S.A. *Canadian Journal of Chemistry* **2001**, *79 (7),* 1115.

2-Formylbenzeneboronic acid (**72**, 0.20 g, 1.33 mmol) was added to a 10 mL round bottom flask and then dissolved in 5.3 mL of previously distilled hexane. Afterwards, 1 equivalent of pinacol (**111**, 0.16 g) and 20% (m/m) of molecular sieves were added and these compound left reacting for 3 h at 25 ºC (first reaction of Scheme **38**, *p.82*). At that time, an extra step of dehydration was performed using dried magnesium sulphate and, then, a vaccum filtration performed. Evaporation of the solvents affored the product (**109** (R = H)) in 95 % yield. The product's characterization was in accordance to what is described on the literature.



^{1}H NMR (400 MHz, $CDCl_3$) δ 10.53 (s, 1H, $C_{arom}CO\underline{H}$), 7.94 (dd, *J* = 7.3, 1.4 Hz, 1H, $C\underline{H}_{arom}$), 7.87 – 7.82 (m, 1H, $C\underline{H}_{arom}$), 7.56 (pd, *J* = 7.4, 1.5 Hz, 2H, $2xC\underline{H}_{arom}$), 1.38 (s, 12H, $C(C\underline{H}_3)_2$).

Step 2 – Methylation of pinacolylboronate ester **109** (R=H)

Literature basis - Vaultier, M.; Rasset-Deloge, C. *Bulletin de la Société Chimique de France* **1994**, *131*, 915.

Compound **109** (R=H, 0.3 g, 1.28 mmol) was then added to a 10 mL round bottom flask and dissolved in 3.8 mL of freshly distilled tetrahydrofuran. Afterwards, 1 equivalent of DBU (0.2 mL) was added to the solution and these compounds reacted for 10 minutes at room temperature. Then, the reaction mixture was cooled down to -78 ºC and once it reached this temperature, 1.1 equivalents methyl lithium (37 µL) was slowly added to the flask. These compounds were left reacting until the reaction warmed to 0 ºC (second reaction of Scheme **38**, *p.82*). At that time, the reaction mixture was acidified until it reached pH 2~4 and a multiple extraction with ether (3x10 mL) was performed. Evaporation of the solvents and a subsequent ^{1}H-NMR evaluation of the crude reaction only showed unreacted compound **109**. After three attemps on making this reaction, the same conclusion was always obtained.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.51 (s, 1H, $C_{arom}CO\underline{H}$), 7.93 – 7.86 (m, 1H, $C\underline{H}_{arom}$), 7.81 (d, *J* = 7.1 Hz, 1H, $C\underline{H}_{arom}$), 7.55 – 7.44 (m, 2H, $2xC\underline{H}_{arom}$), 1.33 (s, 12H, $C(C\underline{H}_3)_2$).

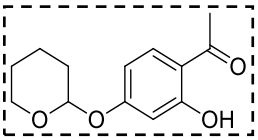
VI.3.1.2. Construction of 2-carbonylbenzeneboronic acid

VI.3.1.2.1. *Strategy 1*

- Synthesis of compound **116**

Literature basis - Fillaut, J.-L.; Andriès, J.; Marwaha, R. D.; Lanoë, P.-H.; Lohio, O.; Toupet, L.; Gareth Williams, J. a. *Journal of Organometallic Chemistry* **2008**, *693(2)*, 228.

In a round bottom flask, 2',4'-dihydroxyacetophenone (**115**, 1.0 g, 6.6 mmol) and 0.2 equivalents of pyridinium *p*-toluenesulfonate (PPTS, 0.33 g) were dissolved in 20 mL of freshly distilled dichloromethane (DCM). Afterwards a 5 mL solution of freshly distilled dichloromethane with 3 equivalents of 3,4-dihydro-α-pyran (**118**, 1.8 mL, 20 mM) was added to the reaction mixture and left reacting at 20 ºC for 5 h (first reaction of Scheme **40**, *p.84*). After that time, the solvent was evaporated and a recrystallization with hot hexane was performed. The product **116** was obtained in 97% yield.

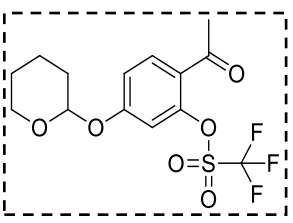


^{1}H NMR (400 MHz, $CDCl_3$) δ 11.86 (d, *J* = 1.5 Hz, 1H, ArO<u>H</u>), 7.36 (d, *J* = 2.4 Hz, 1H, C<u>H</u>$_{arom}$), 7.19 (dd, *J* = 9.0, 2.6 Hz, 1H, C<u>H</u>$_{arom}$), 6.85 (dd, *J* = 9.0, 2.2 Hz, 1H, C<u>H</u>$_{arom}$), 5.26 (s, 1H, -OC<u>H</u>(CH$_2$)O-), 3.88 (t, *J* = 10.3 Hz, 1H, -OC<u>H$_{2a}$</u>CH$_2$-), 3.58 (m, 1H, -OC<u>H$_{2b}$</u>CH$_2$-), 2.56 (s, 3H, C$_{arom}$COC<u>H$_3$</u>), 1.97 – 1.59 (m, 6H, 3xC<u>H$_2$</u>).

- Synthesis of compound **117**

Literature basis - Penhoat, M.; Levacher, V.; Dupas, G. *The Journal of Organic Chemistry* **2003**, *68*, 9517 and Patent: Anacor Pharmaceuticals, Inc.; GlaxoSmithKline; US2010/256092; (**2010**); (A1) English.

In a round bottom flask, compound **116** (1.4 g, 5.9 mmol) and 3 equivalents of freshly distilled triethylamine (TEA, 2.5 mL, 17.6 mmol) were dissolved in 17 mL freshly distilled dichloromethane (DCM) and cooled down to -78 ºC. Afterwards, 1.6 equivalents of triflic anhydride (**119**, 1.8 mL, 20 mM) were slowly added to the reaction mixture and left reacting at -78 ºC for 2 h (second reaction of Scheme **40**, *p.84*). After that time, the reaction mixture was diluted with water and a multiple extraction with dichloromethane (4x10 mL) and brine (4x10 mL) was performed. Then a recrystallization with hot hexane was done to obtain product **117** in 80% yield.



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (dd, *J* = 2.4, 0.9 Hz, 1H, C<u>H</u>$_{arom}$), 7.23 (m, 2H, C<u>H</u>$_{arom}$), 5.46 (t, *J* = 3.0 Hz, 1H, -OC<u>H</u>(CH$_2$)O-), 3.92 – 3.76 (m, 1H, -OC<u>H$_{2a}$</u>CH$_2$-), 3.64 (m, 1H, -OC<u>H$_{2b}$</u>CH$_2$-), 2.61 (s, 3H, C$_{arom}$COC<u>H$_3$</u>), 2.05 – 1.86 (m, 3H, 3xC<u>H$_{2a}$</u>), 1.77 – 1.60 (m, 3H, 3xC<u>H$_{2b}$</u>).

- Synthesis of compound **114**

Literature basis - Anacor Pharmaceuticals, Inc.; GlaxoSmithKline US2010/256092 **2010**.

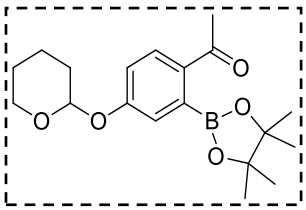
In a flame dried round bottom flask, under inert atmosphere, compound **117** (0.1 g, 0.27 mmol), bis(pinacolato)diboron (**120**, 0.14 g, 0.42 mmol), [1,1'-bis(diphenylphosphino)ferrocene]palladium (II) chloride ($PdCl_2$dppf, 0.022 g, 0.027 mmol) and sodium acetate ($NaCH_3COO$, 0.066 g, 0.81 mmol) were flushed 3 times with Argon. Then, 2 mL of previously distilled dioxane were added and the reaction mixture was heated at 100 ºC for 4 h (Scheme **41**, *p.84*), after which the solvent was concentrated *in vacuo* and the crude purified by preparative chromatography (silica gel, ethyl acetate:hexane [1:4]) to give compound **114** in 40% yield and the side-product (**121**) in 43% yield.

Optimization attempts on Miyaura-borylation:

Literature basis - Ishiyama, T.; Murata, M.; Miyaura, N. *Journal of Organic Chemistry* **1995**, *60*, 7508 and Ishiyama, T.; Itoh, Y.; Kitano, T.; Miyaura, N. *Tetrahedron Letters*, **1997**, *38(19)*, 3447.

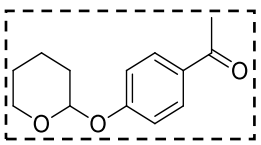
In a flame dried round bottom flask, under inert atmosphere, compound **117** (0.05 g, 0.14 mmol), bis(pinacolato)diboron (**120**, 0.07 g, 0.27 mmol), catalyst (6.8 µmol), ligand (13.6 µmol) and base (0.41 mmol) were flushed 3 times with Argon. Then, 2 mL of previously distilled solvent were added and the reaction mixture was heated at 100 ºC. The reactions were monitored by TLC throughout 8 h. Results and reagents are described in Table **11** (*p.84*).

Boronated product (**114**):



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (d, *J* = 2.0 Hz, 1H, C$\underline{H}_{arom}$), 7.42 (d, *J* = 8.1 Hz, 1H, C$\underline{H}_{arom}$), 7.22 (dd, *J* = 8.1, 2.2 Hz, 1H, C$\underline{H}_{arom}$), 5.46 (s, 1H, -OC$\underline{H}$(CH_2)O-), 3.87 – 3.78 (m, 1H, -OC$\underline{H}_{2a}CH_2$-), 3.63 – 3.54 (m, 1H, -OC$\underline{H}_{2b}CH_2$-), 2.57 (s, 3H, $C_{arom}COC\underline{H}_3$), 2.14 – 1.98 (m, 3H, 3xC$\underline{H}_{2a}$), 1.70 – 1.56 (m, 3H, 3xC$\underline{H}_{2b}$), 1.40 (s, 12H, C($C\underline{H}_3$)$_2$).

Protolytic deboronation product (**121**):



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (s, 1H, C$\underline{H}_{arom}$), 7.58 (d, *J* = 7.6 Hz, 1H, C$\underline{H}_{arom}$), 7.37 (t, *J* = 7.9 Hz, 1H, C$\underline{H}_{arom}$), 7.28 (d, *J* = 2.0 Hz, 1H, C$\underline{H}_{arom}$), 5.49 (d, *J* = 2.8 Hz, 1H, -OC$\underline{H}$(CH_2)O-), 3.95 – 3.82 (m, 1H, -OC$\underline{H}_{2a}CH_2$-), 3.70 – 3.57 (m, 1H, -OC$\underline{H}_{2b}CH_2$-), 2.60 (s, 3H, $C_{arom}COC\underline{H}_3$), 2.08 – 1.86 (m, 3H, 3xC$\underline{H}_{2a}$), 1.79 – 1.53 (m, 3H, 3xC$\underline{H}_{2b}$).

- Synthesis of compounds **113** and **112** (Procedures for deprotection steps)

a) Strong acids

a1 – Hydrochloric acid (HCl)

Literature basis - Martin, R.; Jones, J. B. *Tetrahedron Letters* **1995**, *36*, 8399.

In a flame dried round bottom flask, under inert atmosphere, compound **114** (0.04 g, 0.11 mmol) was dissolved in 1 mL of tetrahydrofuran (THF). After complete dissolution, 1 mL of HCl 5 % was added to the solution and left reacting at 25 ºC for 20 h. Then, the solvents were evaporated under vaccum and a preparative chromatography (silica gel, ethyl acetate:hexane [1:1]) was performed. This procedure afforded vestigial ammounts of compound **113** and the protolytic deboronation product (4-hydroxyacetophenone).

a2 –Acetic acid (CH_3COOH)

Literature basis - *Protective Groups In Organic Synthesis*; Greene, T. W.; Wuts, P. G. M.; Third Ed.; Wiley-VCH, **1999**; Vol. 23.

In a flame dried round bottom flask, under inert atmosphere, compound **114** (0.05 g, 0.14 mmol) was dissolved in 1 mL of dioxane. After complete dissolution, 1 mL of CH_3COOH was added to the solution and left reacting at 25 ºC for 20 h. Then, a multiple extraction with diethyl ether (3x5 mL) and brine (3x5 mL) was performed and the solvents evaporated under vaccum. This procedure afforded vestigial ammounts of compound **113**.

b) Oxidizing agents

Literature basis - Coutts, S. J.; Adams, J.; Krolikowski, D.; Snow, R. J. *Tetrahedron Letters* **1994**, 35, 5109.

In a flame dried round bottom flask, under inert atmosphere, compound **113** (0.05 g, 0.19 mmol) was dissolved in 1.5 mL of THF. After complete dissolution, 1.3 equivalents of $NaIO_4$ (0.05 mg, 0.25 mmol) were added, followed by dilution with 0.2 mL of water. The reaction mixture was left reacting at 25 ºC for 0.5 h. Then, 0.15 mL of 0.1 M HCl was added to this mixture and reacted for 20 h at 25 ºC. Afterwards, a multiple extraction with diethyl ether (3x5 mL) and brine (3x5 mL) was performed and the solvents evaporated under vaccum. The ^{1}H-NMR evaluation of this oil in vestigial ammounts showed a large mixture of unidentified compounds.

c) Methods for Pinacolyl deprotection

c1 – Through trifluoroborate formation

Literature basis – Yuen, A.K.L.; Hutton, C.A.; *Tetrahedron Letters* **2005**, *46*, 7899

In a flame dried round bottom flask, under inert atmosphere, compound **114** (0.07 g, 0.21 mmol) was dissolved in 0.7 mL of methanol. After complete dissolution, a solution of 0.3 mL of water with 5.6 equivalents of potassium hydrogen fluoride (KHF_2, 0.09 mg, 1.16 mmol) was added to the previous solution and left reacting at 25 ºC for 2 h. Then, the solvents were evaporated under vaccum to afford an oil that was dissolved in hot acetone. The solid that did not dissolve was separated with a hot gravity filtration. The solvent was, once again, evaporated under vaccum and a recrystallization with hot acetone and ether afforded the trifluoroborate derivative of compound **114**.

This derivative was then used to perform the last deprotecting step. This compound (42 mg, 0.16 mmol) was dissolved in 1.5 mL of acetonitrile and 3 equivalents of water (9 µL, 0.48 mmol) and, then, 3 equivalents of trimethylsilyl chloride (TMSCl, 61 µL, 0.48 mmol) were added dropwise and left reacting at 25 ºC for 1 h. After which, multiple extraction with 3x1 mL of sodium bicarbonate was performed. Finally, several column chromatographies were performed and compounds **113**, **112** and the protolytic deboronation product were isolated in insignificant yields (lower than 5 %).

c2 – Through complexation with diethylamine

Literature basis – Sun, J.; Perfetti, M.T.; Santos, W.L. *Journal of Organic Chemistry* **2011**; *76(9)*, 3571

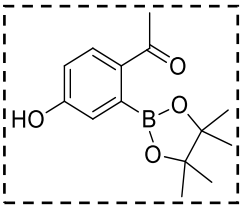
In a flame dried round bottom flask, under inert atmosphere, compound **113** (0.05 g, 0.19 mmol) was dissolved in 1.5 mL of diethyl ether. After complete dissolution, 1.1 equivalents of diethylamine (20 µL, 0.21 mmol) were added and left reacting at 25 ºC for 22 h. At that time, a multiple extaction with brine (3x2 mL) and dichloromethane (3x2 mL) was performed and HCl 0.1 M was added to the aqueous phase to cleave the complexation between diethylamine and the boronic acid. Evaluation of this phase did not afford any separable compound, supposably due to a known complexation with 2-carbonylbenzeneboronic acids (Klis, T.; Serwatowski, J. *Tetrahedron Letters* **2007**, *48,* 5223).

d) Methods for THP deprotection

Literature basis – Miyashita, M.; Yoshikoshi, A.; Grieco P.A. *Journal of Organic Chemistry*, **1977**, *42(23)*, 3772

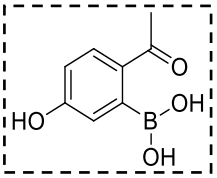
In a flame dried round bottom flask, under inert atmosphere, compound **114** (0.24 g, 0.7 mmol) was dissolved in 5 mL of ethanol. After complete dissolution, 0.1 equivalents of PPTS (18 mg, 0.07 mmol) were added and left reacting at 55 ºC for 4 h. At that time, the solvent was evaporated and a flash column chromatography (silica gel, ethyl acetate:hexane [1:4 - 3:7]) was performed, affording compound **113** in **82**% yield.

- Compound **113**:



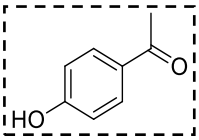
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 (d, *J* = 4.4 Hz, 1H, C$\underline{H}_{arom}$), 7.08 (s, 1H, C$\underline{H}_{arom}$), 6.83 (d, *J* = 7.9 Hz, 1H, C$\underline{H}_{arom}$), 2.41 (s, 3H, $C_{arom}COC\underline{H}_3$), 1.37 (s, 12H, $C(C\underline{H}_3)_2$).

- Compound **112**:



^{1}H NMR (400 MHz, D_2O) δ 7.47 (s, 1H, C$\underline{H}_{arom}$), 7.37 (d, *J* = 8.0 Hz, 1H, C$\underline{H}_{arom}$), 7.14 (d, *J* = 7.9 Hz, 1H, C$\underline{H}_{arom}$), 2.61 (s, 3H, $C_{arom}COC\underline{H}_3$).

- Protolytic deboronation product:



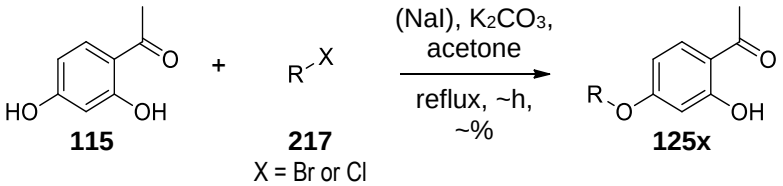
^{1}H NMR (400 MHz, D_2O) δ 7.40 (d, *J* = 8.5 Hz, 2H, 2xC$\underline{H}_{arom}$), 7.13 (d, *J* = 8.5 Hz, 2H, 2xC$\underline{H}_{arom}$), 2.61 (d, *J* = 10.2 Hz, 3H, $C_{arom}COC\underline{H}_3$).

VI.3.1.2.2. *Strategy 2*

- **General Procedures**

Step 1 – Alkylation Procedure

Literature basis - Al-Smadi, M.; Hanold, N.; Kalbitz, H.; Meier, H.; *Synthesis*, **2009**, *15*, 2539

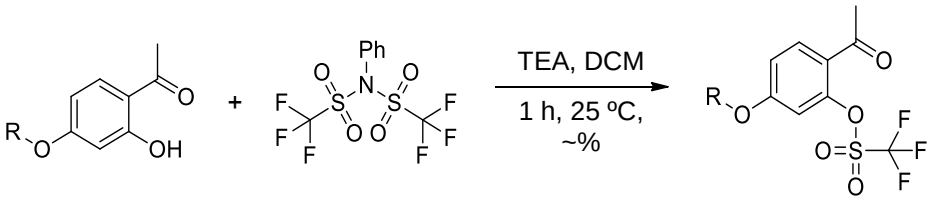


Scheme **77** – General alkylation procedure of compound **115**

In a round bottom flask (A), 2',4'-dihydroxyacetophenone (**115**, 1 equivalent) and potassium carbonate (1 equivalent) were heated in acetone to reflux for 2 h. In a separate round bottom flask (B), haloalkyl derivatives (**217**x, 1 equivalent) and sodium iodide (1.1 equivalents) were stirred in acetone at room temperature for 2 h. Then, flask B was transferred to flask A and refluxed for 20 h (Scheme **77**). After that, acetone was evaporated to dryness and water was added to the reaction mixture. Then, the compound was extracted with diethyl ether. The solution was dried (Na_2SO_4), concentrated and purified by column chromatography (silica gel, hexane:diethyl ether) to give different compounds (**125**x) with variable yields.

Step 2 – Triflation Procedure

Literature basis - Luker, T.; Bonnert, R.; Paine, S.W.; Schmidt, J.; Sargent, C.; Cook, A.R.; Cook, A.; Gardiner, P.; Hill, S.; Weyman-Jones, C.; Patel, A.; Thom, S.; Thorne, P.; *Journal of Medicinal Chemistry* **2011** , *54 (6)*, 1779



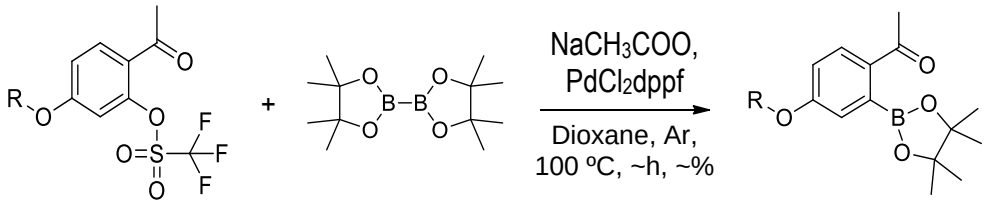
125x **127** **124x**

Scheme **78** – General Triflation of compound **125x**

In a round bottom flask, N-Phenyltrifluoromethanesulfonimide (**127**, 1.2 equivalents) was added portion wise to a solution of the alkylated compound (**125**x, 1 equivalent) with triethylamine (1.5 equivalents) in DMF (0.5 M) and the reaction stirred for 1 h at 25 ºC (Scheme **78**). Water was added and the mixture extracted with diethyl ether. The organic layer was washed with water, LiCl (aq), brine, dried (Na_2SO_4), concentrated and purified by column chromatography (silica gel, hexane:diethyl ether) to give different compounds (**124**x) with variable yields.

Step 3 – Miyaura-Borylation Procedure

Literature basis - Patent: Anacor Pharmaceuticals, Inc.; GlaxoSmithKline; US2010/256092; (**2010**); (A1) English



124x **120** **123x**

Scheme **79** – General Miyaura-borylation of compound **124x**

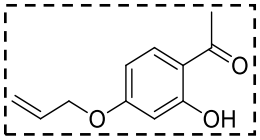
In a flame dried round bottom flask, under inert atmosphere, the triflate derivative (**124**x, 1 equivalent), bis(pinacolato)diboron (**120**, 2 equivalents), [1,1'-bis(diphenylphosphino)ferrocene]palladium (II) chloride ($PdCl_2$dppf, 0.1 equivalents) and sodium acetate ($NaCH_3COO$, 3 equivalents) were flushed 3 times with Argon. Then, previously distilled dioxane (0.15 M) was added and the reaction mixture was heated at 100 ºC for 4 h (Scheme **79**), after which it was concentrated *in vacuo* and the crude purified by flash column cromatography (silica gel, ethyl acetate:hexane) to give different compounds (**123**x) with variable yields.

For the kinetic ^{1}H-NMR evaluations, at pre-determined times, 0.1 mL of the reaction mixture were transferred to a NMR tube and diluted with 0.3 mL of deuterated DMSO. At this point, a ^{1}H-NMR spectrum was collected for each sample.

- **Reactions starting with 3'-bromopropen** (Scheme **44** and Scheme **45**, *p.87*)

Step 1 – Alkylation – Synthesis of compound **125**a

Following the general alkylation procedure (without sodium iodide) and using 3'-bromopropen (**126**, 0.013 mol) as the mentioned haloalkyl derivative, compound **125**a was obtained as a white solid in 90% yield (2.34g).

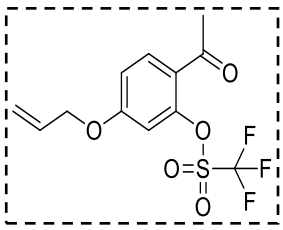


^{1}H NMR (600 MHz, $CDCl_3$) δ 12.71 (s, 1H, $C_{arom}O\underline{H}$), 7.60 (d, *J* = 8.9 Hz, 1H, $C\underline{H}_{arom}$), 6.43 (ddd, *J* = 8.9, 2.3, 1.2 Hz, 1H, $C\underline{H}_{arom}$), 6.38 (d, *J* = 1.4 Hz, 1H, $C\underline{H}_{arom}$), 6.04 – 5.97 (m, 1H, $CH_2C\underline{H}CH_2O$-), 5.40 (d, *J* = 17.3 Hz, 1H, $C\underline{H}_{2a}CHCH_2O$-), 5.30 (d, *J* = 10.5 Hz, 1H, $C\underline{H}_{2b}CHCH_2O$-), 4.54 – 4.52 (m, 2H, $CH_2CHC\underline{H}_2O$-), 2.54 – 2.52 (m, 3H, $C_{arom}COC\underline{H}_3$).

^{13}C NMR (151 MHz, $CDCl_3$) δ 202.72 (s, $C_{arom}\underline{C}OCH_3$), 165.22 (s, $CH\underline{C}_{arom}OHC$), 165.12 (s, $\underline{C}_{arom}OCH_2$-), 132.45 (s, $\underline{C}H_{arom}$), 132.29 (s, $CH_2\underline{C}HCH_2O$-), 118.48 (s, $\underline{C}H_2CHCH_2O$-), 114.07 (s, $CH\underline{C}_{arom}COCH_3$), 108.09 (s, $\underline{C}H_{arom}$), 101.75 (s, $\underline{C}H_{arom}$), 69.05 (s, $CH_2CH\underline{C}H_2O$-), 26.33 (s, $C_{arom}CO\underline{C}H_3$).

Step 2 – Triflation – Synthesis of compound **124**a

Following the general triflation procedure and using compound **125**a as the starting material (0.012 mol), compound **124**a was obtained as a white solid in 93% yield (3.64g).



^{1}H NMR (600 MHz, $CDCl_3$) δ 7.83 (d, *J* = 8.8 Hz, 1H, C<u>H</u>$_{arom}$), 6.96 (dd, *J* = 8.8, 2.4 Hz, 1H, C<u>H</u>$_{arom}$), 6.83 (d, *J* = 2.3 Hz, 1H, C<u>H</u>$_{arom}$), 6.07 – 5.96 (m, 1H, $CH_2C\underline{H}CH_2O$-), 5.43 (dd, *J* = 17.3, 1.1 Hz, 1H, C<u>H$_{2a}$</u>$CHCH_2O$-), 5.36 (dd, *J* = 10.5, 1.2 Hz, 1H, C<u>H$_{2b}$</u>$CHCH_2O$-), 4.65 – 4.58 (m, 2H, $CH_2CHC\underline{H}_2O$-), 2.59 (s, 3H, $C_{arom}COC\underline{H}_3$).

^{13}C NMR (151 MHz, $CDCl_3$) δ 195.21 (s, $C_{arom}\underline{C}OCH_3$), 162.72 (s, $(CH)_2\underline{C}_{arom}OCH_2$), 148.57 (s, $\underline{C}_{arom}OTf$), 132.87 (s, C<u>H</u>$_{arom}$), 131.66 (s, $CH_2\underline{C}HCH_2O$-), 124.10 (s, $CH\underline{C}_{arom}COCH_3$), 119.06 (s, $\underline{C}H_2CHCH_2O$-), 118.72 (q, *J* = 320,1 Hz, -$SO_2\underline{C}F_3$), 114.12 (s, C<u>H</u>$_{arom}$), 109.77 (s, C<u>H</u>$_{arom}$), 69.70 (s, $CH_2CH\underline{C}H_2O$-), 29.13 (s, $C_{arom}CO\underline{C}H_3$).

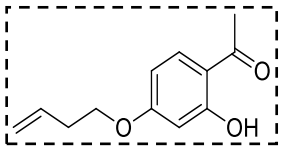
Step 3 – Miyaura-Borylation – Synthesis of compound **123**a (Unsuccessful)

Based on general borylation procedure, several attempts were developed to synthesize compound **123**a, however, all proved to be unsuitable. Among those attempts, experiences with diferent number of equivalents of the starting materials, different temperatures and times, different concentrations and diferent purifications of the starting materials were performed.

- **Reactions starting with 4-bromo-1-butene** (Scheme **46**, *p.87*)

Step 1 – Alkylation – Synthesis of compound **125**b

Following the general alkylation procedure and using 4-bromo-1-butene (**132**, 0.013 mol) as the mentioned haloalkyl derivative, compound **125**b was obtained as a white solid in 44% yield (1.2g).



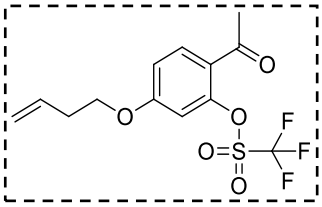
^{1}H NMR (600 MHz, $CDCl_3$) δ 12.73 (s, 1H, $C_{arom}O\underline{H}$), 7.59 (dd, *J* = 8.9, 1.2 Hz, 1H, C<u>H</u>$_{arom}$), 6.44 – 6.37 (m, 2H, 2xC<u>H</u>$_{arom}$), 5.90 – 5.82 (m, 1H, $CH_2C\underline{H}CH_2$-), 5.16 (d, J = 17.2 Hz, 1H, C<u>H$_{2a}$</u>$CHCH_2$-), 5.11 (d, J = 10.3 Hz, 1H, C<u>H$_{2b}$</u>$CHCH_2$-), 4.01 (td, *J* = 6.7, 1.1 Hz, 2H, -$CHCH_2C\underline{H}_2O$-), 2.55 – 2.51 (m, 5H, -$CHC\underline{H}_2CH_2O$- and $C_{arom}COC\underline{H}_3$).

^{13}C NMR (126 MHz, $CDCl_3$) δ 202.59 (s, $C_{arom}\underline{C}OCH_3$), 165.49 (s, $CH\underline{C}_{arom}OHC$), 165.26 (s, $\underline{C}_{arom}OCH_2$-), 133.92 (s, $CH_2\underline{C}HCH_2$-), 132.36 (s, C<u>H</u>$_{arom}$), 117.48 (s, $\underline{C}H_2CHCH_2$-), 113.93 (s,

$CHC_{arom}COCH_3$), 107.97 (s, CH_{arom}), 101.42 (s, CH_{arom}), 67.52 (s, -$CHCH_2CH_2O$-), 33.34 (s, -$CHCH_2CH_2O$-), 26.23 (s, $C_{arom}COCH_3$).

Step 2 – Triflation – Synthesis of compound **124**b

Following the general triflation procedure and using compound **125**b as the starting material (0.01 mol), compound **124**b was obtained as a white solid in 91% yield (3.08g).

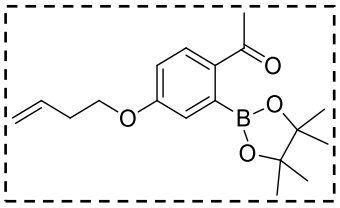


^{1}H NMR (600 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.7 Hz, 1H, CH_{arom}), 6.94 (d, *J* = 8.6 Hz, 1H, CH_{arom}), 6.78 (s, 1H, CH_{arom}), 5.92 – 5.77 (m, 1H, CH_2CHCH_2-), 5.14 (dd, *J* = 29.6, 13.3 Hz, 2H, CH_2CHCH_2-), 4.06 (t, *J* = 4.7 Hz, 2H, -$CHCH_2CH_2O$-), 2.56 (s, 5H, -$CHCH_2CH_2O$- and $C_{arom}COCH_3$).

^{13}C NMR (151 MHz, $CDCl_3$) δ 195.17 (s, $C_{arom}COCH_3$), 163.07 (s, $C_{arom}OCH_2$-), 148.55 (s, $C_{arom}OTf$), 133.53 (s, CH_2CHCH_2-), 132.90 (s, CH_{arom}), 123.83 (s, $CHC_{arom}COCH_3$), 118,69 (q, *J* = 320,9 Hz, -SO_2CF_3), 117.81 (s, CH_2CHCH_2-), 113.73 (s, CH_{arom}), 109.53 (s, CH_{arom}), 68.21 (s, -$CHCH_2CH_2O$-), 33.23 (s, -$CHCH_2CH_2O$-), 29.01 (s, $C_{arom}COCH_3$).

Step 3 – Miyaura-Borylation – Synthesis of compound **123**b

Following the general borylation procedure and using compound **124**b as the starting material (0.3 mmol), compound **123**b was obtained as a yellow solid in 25% yield (0.024g). This reaction was monitored by a ^{1}H-NMR kinetic study following the mentioned general procedure (Figure **29**, *p.88*).



^{1}H NMR (600 MHz, $CDCl_3$) δ 7.76 (d, *J* = 8.6 Hz, 1H, CH_{arom}), 6.96 (d, *J* = 2.3 Hz, 1H, CH_{arom}), 6.85 (dd, *J* = 8.6, 2.4 Hz, 1H, CH_{arom}), 5.93 – 5.84 (m, 1H, CH_2CHCH_2-), 5.16 (d, J = 17.2 Hz, 1H, $CH_{2a}CHCH_2$-), 5.11 (d, J = 10.2 Hz, 1H, $CH_{2b}CHCH_2$-), 4.07 (t, J = 6.6 Hz, 2H, -$CHCH_2CH_2O$-), 2.56 – 2.52 (m, 5H, -$CHCH_2CH_2O$- and $C_{arom}COCH_3$), 1.43 (s, 12H, 2x$C(CH_3)_2$).

^{13}C NMR (151 MHz, $CDCl_3$) δ 198.61 (s, $C_{arom}COCH_3$), 162.74 (s, $C_{arom}OCH_2$-), 134.18 (s, CH_2CHCH_2-), 133.51 (s, $CHC_{arom}COCH_3$), 130.86 (s, CH_{arom}), 118.04 (s, CH_{arom}), 117.43 (s, CH_2CHCH_2-), 114.16 (s, CH_{arom}), 83.71 (s, $C(CH_3)_2$), 67.40 (s, -$CHCH_2CH_2O$-), 33.58 (s, -$CHCH_2CH_2O$-), 25,05 (s, $C(CH_3)_2$), 24.92 (s, $C_{arom}COCH_3$).

^{13}C-HMBC 138.5 (s, $C_{arom}B$)

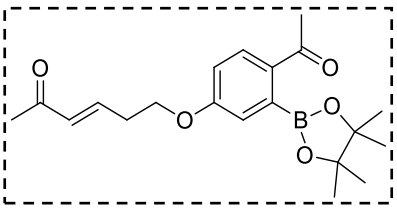
- **General procedure of Cross-Metathesis** (Scheme **47**, *p.88*)

Literature Basis – Voigtritter, K.; Ghorai, S.; Lipshutz, B.H. *Journal of Organic Chemistry* **2011**, *76*, 4697

In a flame dried round bottom flask, under inert atmosphere, compound **123**b (1 equivalent), acrylate/ketone (3 equivalents), Hoveyda-Grubbs 2nd generation catalyst (0.03 equivalents) and copper iodide (0.02 equivalents) were placed under an Argon. Freshly distilled diethyl ether (0.1 M) was added and a reflux condenser was placed on the vial. The reaction mixture was heated at 40 ºC for 3 h (Scheme **47**, *p.88*), after which it was concentrated *in vacuo* and the crude purified by flash column cromatography (silica gel, diethyl ether:hexane) to give different compounds (**133**x) with variable yields.

Synthesis of compound **133**a

Following the general procedure of cross-methathesis and using methyl vinyl ketone as the starting material (0.35 mmol), compound **133**a was obtained as a yellow solid in 65% yield (0.027g).



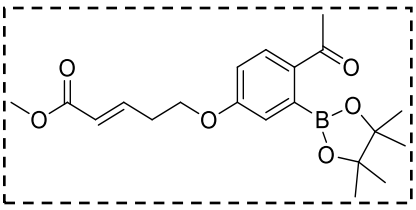
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.6 Hz, 1H, C$\underline{H}_{arom}$), 6.96 (s, 1H, C$\underline{H}_{arom}$), 6.90 – 6.81 (m, 2H, C$\underline{H}_{arom}$, C$\underline{H}$CO), 6.19 (d, *J* = 16.0 Hz, 1H, $CH_2C\underline{H}CH$), 4.16 (t, *J* = 6.3 Hz, 2H, $OC\underline{H}_2CH_2$), 2.72 (q, *J* = 6.3 Hz, 2H, $CH_2C\underline{H}_2CH$), 2.55 (s, 3H, $CHCOC\underline{H}_3$), 2.27 (s, 3H, $C_{arom}COC\underline{H}_3$), 1.43 (s, 12H, $CC\underline{H}_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 198.60 (s, $\underline{C}OCH_3$), 162.32 (s, $\underline{C}_{arom}OCH_2$), 143.33 (s, CH$\underline{C}$HCO), 133.88 (s, CH$\underline{C}$CO), 133.35 (s, $CH_2\underline{C}HCH$), 130.88 (s, C$\underline{H}_{arom}$), 117.96 (s, C$\underline{H}_{arom}$), 114.19 (s, C$\underline{H}_{arom}$), 83.80 (s, $\underline{C}(CH_3)_2$), 66.16 (s, $O\underline{C}H_2CH_2$), 32.29 (s,$CH_2\underline{C}H_2CH$), 27.15 (s, $CHCO\underline{C}H_3$), 25.08 (s, $C(\underline{C}H_3)_2$), 24.99 (s, $C_{arom}CO\underline{C}H_3$).

^{13}C-HMBC 138.7 (s, C_{arom}B)

Synthesis of compound **133**b

Following the general procedure of cross-methathesis and using methyl acrylate as the starting material (0.11 mmol), compound **133**b was obtained as a yellowish solid in 68% yield (0.081 g).



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 8.6 Hz, 1H, CH_{arom}), 7.06 – 6.91 (m, 2H, CH_{arom}, CHCO), 6.83 (d, *J* = 8.6 Hz, 1H, CH_{arom}), 5.95 (d, *J* = 16.0 Hz, 1H, CH_2CHCH), 4.12 (t, *J* = 6.3 Hz, 2H, OCH_2), 3.72 (s, 3H, OCH_3), 2.68 (q, *J* = 6.3 Hz, 2H, CH_2CH), 2.53 (s, 3H, COCH_3), 1.42 (s, 12H, 2xC(CH_3)$_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 198.50 (s, C_{arom}COCH$_3$), 166.70 (s, CHCOOCH$_3$), 162.26 (s, C_{arom}OCH$_2$), 144.53 (s, CHCHCO), 133.73 (s, CH$_3$COC_{arom}), 130.81 (s, CH_{arom}), 123.31 (s, CH$_2$CHCH), 117.87 (s, CH_{arom}), 114.12 (s, CH_{arom}), 83.68 (s, C(CH$_3$)$_2$), 66.03 (s, OCH$_2$CH$_2$), 51.62 (s, COOCH$_3$), 31.93 (s, CH$_2$CH$_2$CH), 25.00 (s, C(CH$_3$)$_2$), 24.90 (s, CH$_3$COC$_{arom}$)

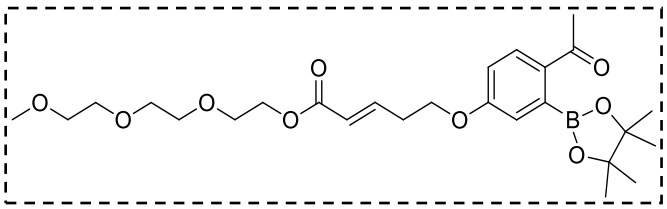
^{13}C-HMBC 138.7 (s, C$_{arom}$B)

Synthesis of compound **133**c (Unsuccessful)

Following the general procedure of cross-methathesis and using a synthesized dansyl derivative (*N*-[(5-*N,N*-Dimethylaminonaphthylsulfonylamino)ethyl]-2-methacrylamide, Literature Basis – Sun, W.; Bandmann, H.; Schrader, T. *Chemistry – A European Journal* **2007**, *13*, 7701), three attempts were made to produce compound **133**c though all proved to be unsuccessful.

Synthesis of compound **133**d

Triethylene glycol monomethyl ether acrylate was synthesized according to a procedure described in the literature (Ryu, J.-H.; Roy, R.; Ventura, J.; Thayumanavan, S. *Langmuir,* **2010**, *26(10)*, 7086) and applied to the mentioned general procedure of cross-methathesis. This reaction afforded compound **133**d was obtained as a colorless liquid in 60% yield (0.165 g *trans* isomer and 0.008 g of *cis* isomer).



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 8.6 Hz, 1H, CH_{arom}), 7.03 (d, *J* = 15.7 Hz, 1H, CHCO), 6.95 (d, *J* = 1.9 Hz, 1H, CH_{arom}), 6.85 (dd, *J* = 8.6, 1.9 Hz, 1H, CH_{arom}), 5.99 (d, *J* = 15.7 Hz, 1H, CH_2CHCH), 4.32 – 4.28 (m, 2H, OCH_2), 4.14 (t, *J* = 6.3 Hz, 2H, OCH_2), 3.75 – 3.71 (m, 2H, OCH_2), 3.69 – 3.62 (m, 6H, OCH_2), 3.56 – 3.52 (m, 2H, OCH_2), 3.37 (s, 3H, OCH_3), 2.71 (t, *J* = 6.4 Hz, 2H, 2H, CH_2CH), 2.55 (s, 3H, COCH_3), 1.43 (s, 12H, 2xC(CH_3)$_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 198.60 (s, C_{arom}COCH$_3$), 166.29 (s, CHCOOCH$_3$), 162.34 (s, C_{arom}OCH$_2$), 144.81 (s, CHCHCO), 133.82 (s, CH$_3$COC_{arom}), 130.88 (s, CH_{arom}), 123.47 (s, CH$_2$CHCH),

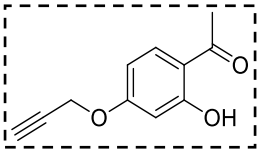
117.90 (s, $\underline{C}H_{arom}$), 114.26 (s, $\underline{C}H_{arom}$), 83.78 (s, $\underline{C}(CH_3)_2$), 72.04 (s, $O\underline{C}H_2$), 70.74 (s, $O\underline{C}H_2$), 70.70 (s, $O\underline{C}H_2$), 69.31 (s, $O\underline{C}H_2$), 66.08 (s, $O\underline{C}H_2$), 66.00 (s, $O\underline{C}H_2$), 63.71 (s, $O\underline{C}H_2$), 59.19 (s, $O\underline{C}H_3$), 32.04 (s, $CH_2\underline{C}H_2CH$), 25.08 (s, $C(\underline{C}H_3)_2$), 24.99 (s, $C_{arom}CO\underline{C}H_3$).

^{13}C-HMBC 138.4 (s, $C_{arom}B$)

- **Reactions starting with propargyl bromide** (Scheme **48**, *p.89*)

Step 1 – Alkylation – Synthesis of compound **125**c

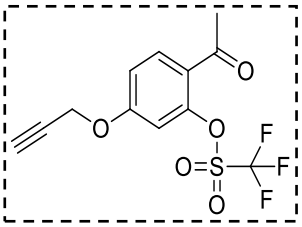
Following the general alkylation procedure and using propargyl bromide (**134**, 6.6 mmol) as the mentioned haloalkyl derivative, compound **125**c was obtained as a white solid in 68% yield (0.85 g).



^{1}H NMR (500 MHz, $CDCl_3$) δ 11.89 (s, 1H, $C_{arom}O\underline{H}$), 7.32 (d, *J* = 3.0 Hz, 1H, $C\underline{H}_{arom}$), 7.18 (dd, *J* = 9.1, 3.0 Hz, 1H, $C\underline{H}_{arom}$), 6.94 (d, *J* = 9.1 Hz, 1H, $C\underline{H}_{arom}$), 4.68 (d, *J* = 2.4 Hz, 2H, $CHCC\underline{H}_2O$-), 2.62 (s, 3H, $C_{arom}COC\underline{H}_3$), 2.54 (t, *J* = 2.4 Hz, 1H, $C\underline{H}CCH_2O$-).

Step 2 – Triflation – Synthesis of compound **124**c

Following the general triflation procedure and using compound **125**c as the starting material (0.42 mmol), compound **124**c was obtained as a white solid in 94% yield (0.12 g).



^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36 (d, *J* = 3.1 Hz, 1H, $C\underline{H}_{arom}$), 7.29 – 7.25 (m, 1H, $C\underline{H}_{arom}$), 7.15 (dd, *J* = 9.0, 3.1 Hz, 1H, $C\underline{H}_{arom}$), 4.75 (d, *J* = 2.4 Hz, 2H, $CHCC\underline{H}_2O$-), 2.61 (s, *J* = 9.9 Hz, 3H, $C_{arom}COC\underline{H}_3$), 2.58 (s, *J* = 2.4 Hz, 1H, $C\underline{H}CCH_2O$-).

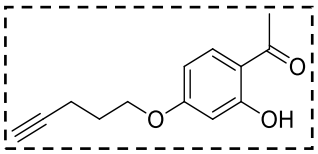
Step 3 – Miyaura-Borylation – Synthesis of compound **123**c (Unsuccessful)

Based on general borylation procedure, two attempts were made to synthesize compound **123**c, however, after a few minutes of reaction the starting material **124**c was fully consumed without forming any new relevant spot on the TLC.

- **Reactions starting with 5-chloro-1-pentyne** (Scheme **49**, *p.89*)

Step 1 – Alkylation – Synthesis of compound **125**d

Following the general alkylation procedure and using 5-chloro-1-pentyne (**135**, 6.6 mmol) as the mentioned haloalkyl derivative, after 28 h, compound **125**d was obtained as a white solid in 62% yield (0.9 g).

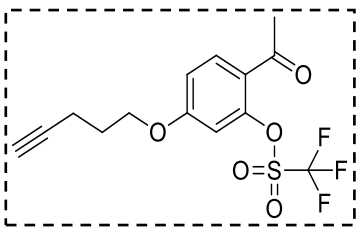


^{1}H NMR (500 MHz, $CDCl_3$) δ 12.72 (s, 1H, $C_{arom}O\underline{H}$), 7.62 (d, *J* = 8.7 Hz, 1H, $C\underline{H}_{arom}$), 6.46 – 6.39 (m, 2H, 2x$C\underline{H}_{arom}$), 4.10 (t, *J* = 6.1 Hz, 2H, -$CH_2CH_2C\underline{H_2}O$-), 2.55 (s, 3H, $C_{arom}COC\underline{H_3}$), 2.40 (td, *J* = 6.9, 2.6 Hz, 2H, -$CH_2C\underline{H_2}CH_2$-), 2.05 – 1.97 (m, 3H, $C\underline{H}CC\underline{H_2}CH_2$-).

^{13}C NMR (151 MHz, $CDCl_3$) δ 202.70 (s, $C_{arom}\underline{C}OCH_3$), 165.51 (s, $\underline{C}_{arom}OCH_2$-), 165.31 (s, $CH\underline{C}_{arom}OHC$), 132.43 (s, $\underline{C}H_{arom}$), 114.01 (s, -$CH\underline{C}_{arom}COCH_3$), 108.02 (s, $\underline{C}H_{arom}$), 101.47 (s, $\underline{C}H_{arom}$), 83.14 (s, $CH\underline{C}CH_2CH_2$-), 69.31 (s, $\underline{C}HCCH_2CH_2$-), 66.54 (s, -$CH_2CH_2\underline{C}H_2O$-), 27.91 (s, $CHC\underline{C}H_2CH_2$-), 26.36 (s, $C_{arom}CO\underline{C}H_3$), 15.20 (s, -$CH_2\underline{C}H_2CH_2$-).

Step 2 – Triflation – Synthesis of compound **124**d

Following the general triflation procedure and using compound **125**d as the starting material (0.42 mmol), compound **124**d was obtained as a white solid in 90% yield (1.2 g).



^{1}H NMR (600 MHz, $CDCl_3$) δ 7.84 (d, *J* = 8.8 Hz, 1H, $C\underline{H}_{arom}$), 6.96 (dd, *J* = 8.8, 2.4 Hz, 1H, $C\underline{H}_{arom}$), 6.82 (d, *J* = 2.2 Hz, 1H, $C\underline{H}_{arom}$), 4.15 (t, *J* = 6.1 Hz, 2H, -$CH_2CH_2C\underline{H_2}O$-), 2.59 (s, 3H, $C_{arom}COC\underline{H_3}$), 2.42 (td, *J* = 6.8, 2.7 Hz, 2H, -$CH_2C\underline{H_2}CH_2$-), 2.13 – 1.91 (m, 3H, $C\underline{H}CC\underline{H_2}CH_2$-).

^{13}C NMR (151 MHz, $CDCl_3$) δ 195.20 (s, $C_{arom}\underline{C}OCH_3$), 163.05 (s, $\underline{C}_{arom}OCH_2$-), 148.62 (s, $\underline{C}_{arom}OTf$), 132.89 (s, $\underline{C}H_{arom}$), 124.10 (s, $\underline{C}_{arom}COCH_3$), 118.73 (q, *J* = 320.7 Hz, - $SO_2\underline{C}F_3$), 113.79 (s, $\underline{C}H_{arom}$), 109.51 (s, $\underline{C}H_{arom}$), 82.83 (s, $CH\underline{C}CH_2CH_2$-), 69.53 (s, $\underline{C}HCCH_2CH_2$-), 67.17 (s, -$CH_2CH_2\underline{C}H_2O$-), 29.23 (s, $CHC\underline{C}H_2CH_2$-), 27.75 (s, $C_{arom}CO\underline{C}H_3$), 15.11 (s, -$CH_2\underline{C}H_2CH_2$-).

Step 3 – Miyaura-Borylation – Synthesis of compound **123**d (Unsuccessful)

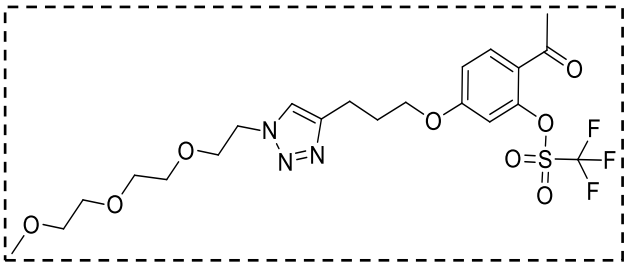
Based on general borylation procedure, two attempts were made to synthesize compound **123**d, however, after a few minutes of reaction the starting material **124**c was also fully consumed without forming any new relevant spot on the TLC. This decomposition was later demonstrated by performing a ^{1}H-NMR of the crude reaction after 2 h (Figure **30**, *p.90*).

- **Alternative substrate for Miyaura-Borylation starting with 5-chloro-1-pentyne** (Scheme **50**, *p.90*)

Step 1 – Synthesis of compound **137**

Literature Basis – Shao, C.; Wang, X.; Zhang, Q.; Luo, S.; Zhao, J.; Hu, Y. *Journal of Organic Chemistry* **2011**, *76*, 6832

In a flame dried round bottom flask, under inert atmosphere, compound **124d** (65 mg, 0.2 mmol), copper iodide (0.4 mg, 1.8 µmol), *N,N*-Diisopropylethylamine (DIPEA, 0.7 µL, 3.7 µmol) and acetic acid (HOAc, 0.2 µL, 3.7 µmol) were mixed in 1 mL of freshly distilled dichloromethane. Then, compound **136** (37 mg, 0.2 mmol) was added at room temperature and left reacting for 5 h (first reaction of Scheme **50**, *p.90*). The reaction mixture was concentrated and purified through a flash chromatography column (silica gel, dichloromethane) to afford 98 mg of compound **137** as a greenish oil (98%).



^{1}H NMR (600 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.8 Hz, 1H, C<u>H</u>$_{arom}$), 7.54 (s, 1H, -NC<u>H</u>C-), 6.94 (d, *J* = 8.8 Hz, 1H, C<u>H</u>$_{arom}$), 6.80 (s, 1H, C<u>H</u>$_{arom}$), 4.51 (t, *J* = 4.9 Hz, 2H, -OCH$_2$C<u>H$_2$</u>N-), 4.10 (t, *J* = 6.1 Hz, 2H, -CH$_2$CH$_2$C<u>H$_2$</u>O-), 3.85 (t, *J* = 4.9 Hz, 2H, -OC<u>H$_2$</u>CH$_2$N-), 3.60 (dd, *J* = 6.6, 3.8 Hz, 6H, -OC<u>H$_2$</u>-), 3.53 (dd, *J* = 5.1, 3.7 Hz, 2H, -OC<u>H$_2$</u>-), 3.36 (s, 3H, -OC<u>H$_3$</u>), 2.91 (t, *J* = 7.4 Hz, 2H, -CC<u>H$_2$</u>CH$_2$-), 2.58 (s, 3H, C$_{arom}$COC<u>H$_3$</u>), 2.26 – 2.19 (m, 2H, -CH$_2$C<u>H$_2$</u>CH$_2$-).

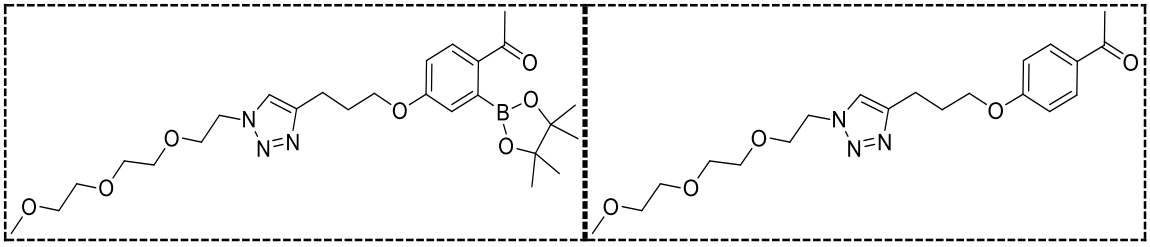
^{13}C NMR (151 MHz, $CDCl_3$) δ 195.18 (s, C$_{arom}$<u>C</u>OCH$_3$), 163.16 (s, <u>C</u>$_{arom}$OCH$_2$-), 148.60 (s, <u>C</u>$_{arom}$OTf), 146.64 (s, -NCH<u>C</u>CH$_2$-), 132.91 (s, <u>C</u>H$_{arom}$), 123.98 (s, <u>C</u>$_{arom}$COCH$_3$), 122.39 (s, -N<u>C</u>HC-), 118.72 (q, *J* = 320.7 Hz, -SO$_2$<u>C</u>F$_3$), 113.68 (s, <u>C</u>H$_{arom}$), 109.62 (s, <u>C</u>H$_{arom}$), 72.00 (s, -O<u>C</u>H$_2$-), 70.61 (s, -O<u>C</u>H$_2$-), 70.57 (s, -O<u>C</u>H$_2$-), 69.63 (s, -O<u>C</u>H$_2$CH$_2$N-), 68.08 (s, -CH$_2$CH$_2$<u>C</u>H$_2$O-), 59.15 (s, -O<u>C</u>H$_3$), 50.26 (s, -OCH$_2$<u>C</u>H$_2$N-), 29.22 (s, C$_{arom}$COC<u>H$_3$</u>), 28.66 (s, -CH$_2$<u>C</u>H$_2$CH$_2$-), 22.02 (s, -C<u>C</u>H$_2$CH$_2$-).

Step 2 – Miyaura-Borylation with compound **137** (resulting from CuAAC)

Literature basis – Patent: Anacor Pharmaceuticals, Inc.; GlaxoSmithKline; US2010/256092; (**2010**); (A1) English

In a flame dried round bottom flask, under inert atmosphere, compound **137** (93 mg, 0.17 mmol), bis(pinacolato)diboron (**120**, 88 mg, 0.35 mmol), [1,1'-bis(diphenylphosphino)ferrocene]palladium (II) chloride (14 mg; 0.017 mmol) and sodium acetate (42 mg, 0.52 mmol) were flushed 3 times with Argon.

Then, 2 mL of previously distilled dioxane were added and degassed for 10 minutes with bubbling Argon. The reaction mixture was heated at 100 ºC for 8 h (second reaction of Scheme **50**, *p.90*), after which the solvent was concentrated in vacuum and the crude residue purified by flash column chromatography (silica gel, dichloromethane:methanol (1:0 – 9.5:0.5)) to give a light yellow oil (42 mg). This oil was a mixture of compound **138** (44%) and the protolytic deboronation compound (56%).



Signals from the protolytic deboronation compound are marked with (*)

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.89 (d, *J* = 8.2 Hz, 1.28H, 2xCH_{arom}*), 7.74 (d, *J* = 8.6 Hz, 1H, CH_{arom}), 7.51 (s, 0.59H, -NC<u>H</u>C-*), 7.49 (s, 1H, -NC<u>H</u>C-), 6.93 (d, *J* = 1.6 Hz, 1H, CH_{arom}), 6.89 (d, *J* = 8.4 Hz, 1.28H, CH_{arom}*), 6.83 (dd, *J* = 8.4, 2.1 Hz, 1H, CH_{arom}), 4.48 (t, *J* = 5.0 Hz, 3.42H, -OCH_2CH_2N- and -OCH_2CH_2N-*), 4.06 (q, *J* = 5.7 Hz, 3.38H, -$CH_2CH_2CH_2$O- and , -$CH_2CH_2CH_2$O-*), 3.82 (t, *J* = 4.9 Hz, 3.37H, -OCH_2CH_2N- and -OCH_2CH_2N-*), 3.61 – 3.53 (m, 10.12H, , -OCH_2- and , -OCH_2-*), 3.50 (dd, *J* = 5.0, 3.8 Hz, 3.29H, , -OCH_2- and , -OCH_2-*), 3.34 (d, *J* = 0.7 Hz, 5.11H, , -OCH_3 and -OCH_3*), 2.89 (dd, *J* = 13.0, 6.7 Hz, 3.47H, , -CCH_2CH_2- and , -CCH_2CH_2-*), 2.52 (s, 4.86H, $C_{arom}COCH_3$ and $C_{arom}COCH_3$*), 2.18 (dd, *J* = 13.5, 6.6 Hz, 3.45H, -$CH_2CH_2CH_2$- and -$CH_2CH_2CH_2$-*), 1.41 (s, 12H, 2x$C(CH_3)_2$).

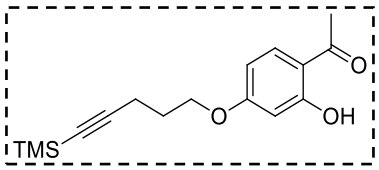
^{13}C NMR (151 MHz, $CDCl_3$) δ 198.51 (s, $C_{arom}COCH_3$), 196.88 (s, $C_{arom}COCH_3$*), 162.97 (s, $C_{arom}OCH_2$-), 162.76 (s, $C_{arom}OCH_2$-*), 146.84 (s, -NCHCCH$_2$-), 146.83 (s, -NCHCCH$_2$-*), 133.42 (s, $C_{arom}COCH_3$), 130.85 (s, CH_{arom}), 130.65 (s, CH_{arom}*), 130.26 (s, $C_{arom}COCH_3$*), 122.31 (s, -NCHC-), 122.29 (s, -NCHC-*), 118.00 (s, CH_{arom}), 114.19 (s, CH_{arom}), 113.99 (s, CH_{arom}*), 83.65 (s, $C(CH_3)_2$), 71.95 (s, -OCH_2-), 70.56 (s, -OCH_2-), 70.53 (s, -OCH_2-), 69.60 (s, -OCH_2CH_2N-), 67.19 (s, -$CH_2CH_2CH_2$O-), 67.04 (s, -$CH_2CH_2CH_2$O-*), 59.11 (s, -OCH_3), 50.16 (s, -OCH_2CH_2N-), 28.83 (s, -$CH_2CH_2CH_2$-), 28.81 (s, -$CH_2CH_2CH_2$-*), 26.43 (s, $C_{arom}COCH_3$*), 25.01 (s, $C(CH_3)_2$), 24.89 (s, $C_{arom}COCH_3$), 22.11 (s, -CCH_2CH_2-), 22.06 (s, -CCH_2CH_2-*).

^{13}C-HMBC 138.5 (s, C_{arom}B)

- **Reactions starting with (5-chloro-1-pentynyl)trimethylsilane** (Scheme **51,** *p.91*)

Step 1 – Alkylation – Synthesis of compound **125**e

Following the general alkylation procedure and using (5-chloro-1-pentynyl)trimethylsilane (**139**, 6.6 mmol) as the mentioned haloalkyl derivative, after 40 h, compound **125**e was obtained as a white solid in 39% yield (0.7 g).

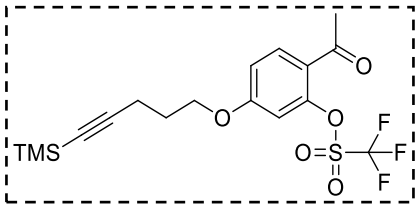


^{1}H NMR (600 MHz, $CDCl_3$) δ 12.72 (s, 1H, $C_{arom}O\underline{H}$), 7.60 (d, *J* = 8.8 Hz, 1H, $C\underline{H}_{arom}$), 6.43 – 6.38 (m, 2H, 2x$C\underline{H}_{arom}$), 4.07 (t, *J* = 6.1 Hz, 2H, -$CH_2CH_2C\underline{H}_2O$-), 2.53 (s, 3H, $C_{arom}COC\underline{H}_3$), 2.41 (t, *J* = 6.9 Hz, 2H, -$CH_2C\underline{H}_2CH_2$-), 1.98 (p, *J* = 6.5 Hz, 2H, $(CH_3)_3SiCCC\underline{H}_2CH_2$-), 0.13 (s, 9H, $(C\underline{H}_3)_3SiCCCH_2CH_2$-).

^{13}C NMR (151 MHz, $CDCl_3$) δ 202.64 (s, $C_{arom}\underline{C}OCH_3$), 165.54 (s, $\underline{C}_{arom}OCH_2$-), 165.31 (s, $CH\underline{C}_{arom}OHC$), 132.40 (s, $\underline{C}H_{arom}$), 113.95 (s, -$CH\underline{C}_{arom}COCH_3$), 108.01 (s, $\underline{C}H_{arom}$), 105.83 (s, $(CH_3)_3SiC\underline{C}CH_2$-), 101.44 (s, $\underline{C}H_{arom}$), 85.60 (s, $(CH_3)_3Si\underline{C}CCH_2$-), 66.69 (s, -$CH_2CH_2\underline{C}H_2O$-), 28.05 (s, $(CH_3)_3SiCC\underline{C}H_2CH_2$-), 26.32 (s, $C_{arom}CO\underline{C}H_3$), 16.60 (s, -$CH_2\underline{C}H_2CH_2$-), 0.21 (s, $(\underline{C}H_3)_3SiCCCH_2CH_2$-).

Step 2 – Triflation – Synthesis of compound **124**e

Following the general triflation procedure and using compound **125**e as the starting material (2.6 mmol), compound **124**e was obtained as a white solid in 82% yield (0.9 g).

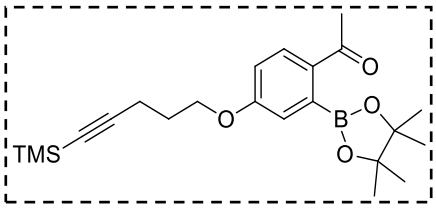


^{1}H NMR (600 MHz, $CDCl_3$) δ 7.83 (d, *J* = 8.8 Hz, 1H, $C\underline{H}_{arom}$), 6.95 (dd, *J* = 8.8, 2.3 Hz, 1H, $C\underline{H}_{arom}$), 6.80 (d, *J* = 2.2 Hz, 1H, $C\underline{H}_{arom}$), 4.13 (t, *J* = 6.1 Hz, 2H, -$CH_2CH_2C\underline{H}_2O$-), 2.58 (s, 3H, $C_{arom}COC\underline{H}_3$), 2.44 (t, *J* = 6.9 Hz, 2H, -$CH_2C\underline{H}_2CH_2$-), 2.01 (p, *J* = 6.5 Hz, 2H, $(CH_3)_3SiCCC\underline{H}_2CH_2$-), 0.13 (s, 9H, $(C\underline{H}_3)_3SiCCCH_2CH_2$-).

^{13}C NMR (151 MHz, $CDCl_3$) δ 195.16 (s, $C_{arom}\underline{C}OCH_3$), 163.12 (s, $\underline{C}_{arom}OCH_2$-), 148.64 (s, $\underline{C}_{arom}OTf$), 132.88 (s, $\underline{C}H_{arom}$), 124.03 (s, -$CH\underline{C}_{arom}COCH_3$), 118.73 (q, *J* = 320.6 Hz, - $SO_2\underline{C}F_3$), 113.79 (s, $\underline{C}H_{arom}$), 109.47 (s, $\underline{C}H_{arom}$), 105.43 (s, $(CH_3)_3SiC\underline{C}CH_2$-), 85.92 (s, $(CH_3)_3Si\underline{C}CCH_2$-), 67.35 (s, -$CH_2CH_2\underline{C}H_2O$-), 29.19 (s, $(CH_3)_3SiCC\underline{C}H_2CH_2$-), 27.85 (s, $C_{arom}CO\underline{C}H_3$), 16.51 (s, -$CH_2\underline{C}H_2CH_2$-), 0.14 (s, $(\underline{C}H_3)_3SiCCCH_2CH_2$-).

Step 3 – Miyaura-Borylation – Synthesis of compound **123**e

Following the general borylation procedure and using compound **124**e as the starting material (0.24 mmol), compound **123**e was obtained as a white solid in 48% yield (46 mg). This reaction was monitored by a 1H-NMR kinetic study following the mentioned general procedure (Figure **31**, *p.91*).



^{1}H NMR (600 MHz, $CDCl_3$) δ 7.77 (d, *J* = 8.6 Hz, 1H, C$\underline{H}_{arom}$), 6.96 (d, *J* = 2.4 Hz, 1H, C$\underline{H}_{arom}$), 6.86 (dd, *J* = 8.6, 2.4 Hz, 1H, C$\underline{H}_{arom}$), 4.11 (t, *J* = 6.1 Hz, 2H, -$CH_2CH_2C\underline{H}_2$O-), 2.55 (s, 3H, $C_{arom}COC\underline{H}_3$), 2.43 (t, *J* = 7.0 Hz, 2H, -$CH_2C\underline{H}_2CH_2$-), 1.99 (t, *J* = 6.5 Hz, 2H, $(CH_3)_3SiCCC\underline{H}_2CH_2$-), 1.43 (s, 12H, 2xC($C\underline{H}_3$)$_2$), 0.13 (s, 9H, $(C\underline{H}_3)_3SiCCCH_2CH_2$-).

^{13}C NMR (151 MHz, $CDCl_3$) δ 198.66 (s, $C_{arom}\underline{C}OCH_3$), 162.77 (s, $\underline{C}_{arom}OCH_2$-), 133.52 (s, -CH$\underline{C}_{arom}COCH_3$), 130.89 (s, $\underline{C}H_{arom}$), 118.14 (s, $\underline{C}H_{arom}$), 114.01 (s, $\underline{C}H_{arom}$), 106.02 (s, $(CH_3)_3SiC\underline{C}CH_2$-), 85.51 (s, $(CH_3)_3Si\underline{C}CCH_2$-), 83.73 (s, $\underline{C}(CH_3)_2$), 66.52 (s, -$CH_2CH_2\underline{C}H_2O$-), 28.23 (s, $(CH_3)_3SiCC\underline{C}H_2CH_2$-), 25.05 (s, $C(\underline{C}H_3)_2$), 24,92 (s, $C_{arom}CO\underline{C}H_3$), 16.65 (s, -$CH_2\underline{C}H_2CH_2$-), 0.23 (s, $(\underline{C}H_3)_3SiCCCH_2CH_2$-).

^{13}C-HMBC 138.6 (s, $\underline{C}_{arom}$B)

Step 4 – Deprotection – Synthesis of compound **123**e1 (Unsuccessful)

Literature Basis – Cai, C.; Vasella, A. *Helvetica Chimica Acta* **1995**, *78(3)*, 732

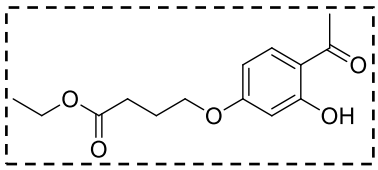
In a flame dried round bottom flask, under inert atmosphere, compound **123**e (30 mg, 0.075 mmol) was dissolved in 0.5 mL of THF at 0 ºC. Then a solution of 0.7 mL of THF (with 5% water) with tetrabutylammonium fluoride trihydrate (30 mg, 0.095 mmol) was added and stirred for 5 minutes. Then, 1.2 mL hexane was added and it was left stirring for more 10 minutes. After that, the reaction mixture was concentrated under reduced pressure and a preparative chromatography was performed (silica gel, hexane:diethyl ether (3:2)) to afford the starting material as the minor product and a product without alkylic proton signals as the major product. No expected product was ever detected.

VI.3.1.2.3. *Strategy 3*

- Synthesis of compound **143**

Literature basis - Al-Smadi, M.; Hanold, N.; Kalbitz, H.; Meier, H.; *Synthesis*, **2009**, *15*, 2539

In a round bottom flask (A), 2',4'-dihydroxyacetophenone (**115**, 5.0 g, 32.9 mmol) and potassium carbonate (4.5 g, 32.9 mmol) were heated in acetone to reflux for 2 h. In a separate round bottom flask (B), ethyl 4-bromobutyrate (**145**, 4.7 mL, 32.9 mmol) and sodium iodide (5.4 g, 36.1 mmol) were stirred in acetone at room temperature for 2 h. Then, flask B was transferred to flask A and refluxed for 20 h (first reaction of Scheme **53**, *p.93*). After that, acetone was evaporated to dryness and 200 mL of water were added and extracted with diethyl ether (3*200 mL). The solution was dried (Na_2SO_4), concentrated and purified by column chromatography (silica gel, hexane:diethyl ether (9:1)) to give a viscous oil that solidified below 20 ºC; compound **143** (7.6 g, 87%)



^{1}H NMR (400 MHz, $CDCl_3$): δ 12.70 (s, 1H, $C_{arom}O\underline{H}$), 7.59 (d, *J* = 8.9 Hz, 1H, $C\underline{H}_{arom}$), 6.39 (d, *J* = 8.9 Hz, 1H, $C\underline{H}_{arom}$), 6.36 (s, 1H, $C\underline{H}_{arom}$), 4.12 (q, *J* = 7.1 Hz, 2H, $OC\underline{H}_2CH_3$), 4.02 (t, *J* = 6.1 Hz, 2H, $OC\underline{H}_2CH_2$), 2.52 (s, 3H, $COC\underline{H}_3$), 2.48 (t, *J* = 7.2 Hz, 2H, $CH_2C\underline{H}_2CO$), 2.09 (p, *J* = 6.6 Hz, 2H, $CH_2C\underline{H}_2CH_2$), 1.24 (t, *J* = 7.1 Hz, 3H, $CH_2C\underline{H}_3$).

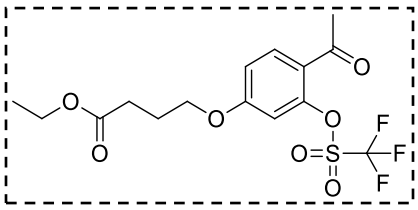
^{13}C NMR (101 MHz, $CDCl_3$): δ 202.62 (s, $C\underline{C}OCH_3$), 173.01 (s, $CH_2\underline{C}OO$), 165.37 (s, $CH\underline{C}_{arom}OHC$), 165.21 (s, $(CH)_2\underline{C}_{arom}OCH_2$), 132.39 (s, $\underline{C}H_{arom}$), 113.95 (s, $CH\underline{C}_{arom}COCOH$), 107.85 (s, $\underline{C}H_{arom}$), 101.42 (s, $\underline{C}H_{arom}$), 67.15 (s, $O\underline{C}H_2CH_2$), 60.59 (s, $O\underline{C}H_2CH_3$), 30.68 (s, $CH_2\underline{C}H_2CO$), 26.28 (s, $CO\underline{C}H_3$), 24.39 (s, $CH_2\underline{C}H_2CH_2$), 14.29 (s, $CH_2\underline{C}H_3$).

HRMS (ESI): *m/z* calculated $[M+Na]^+$ = 289.10464, found $[M+Na]^+$ = 289.10334.

- Synthesis of compound **144**

Literature basis - Luker, T.; Bonnert, R.; Paine, S.W.; Schmidt, J.; Sargent, C.; Cook, A.R.; Cook, A.; Gardiner, P.; Hill, S.; Weyman-Jones, C.; Patel, A.; Thom, S.; Thorne, P.; *Journal of Medicinal Chemistry* **2011** , *54 (6)*, 17

In a round bottom flask, N-Phenyltrifluoromethanesulfonimide (**127**, 3.05 g, 8.5 mmol) was added portion wise to a solution of compound **143** (3.0 g, 11.3 mmol) and triethylamine (TEA, 4.7 mL, 33.8 mmol) in DMF (27 mL) and the reaction stirred for 2 h at 25 ºC (second reaction of Scheme **53**, *p.93*). After that, the mixture mixture was diluted in water and extracted with diethyl ether. The organic layer was washed with water, LiCl (aq), brine, dried (Na_2SO_4), concentrated and purified by column chromatography (silica gel, hexane:diethyl ether (4:1 – 1:1)) to give compound **144** as a white solid (3.9 g, 90%).



^{1}H NMR (400 MHz, $CDCl_3$): δ 7.82 (d, *J* = 8.8 Hz, 1H, C<u>H</u>$_{arom}$), 6.93 (d, *J* = 8.8 Hz, 1H, C<u>H</u>$_{arom}$), 6.79 (s, 1H, C<u>H</u>$_{arom}$), 4.14 (q, *J* = 7.1 Hz, 2H, OC<u>H</u>$_2$CH$_3$), 4.08 (t, *J* = 6.1 Hz, 2H, OC<u>H</u>$_2$CH$_2$), 2.57 (s, 3H, COC<u>H</u>$_3$), 2.50 (t, *J* = 7.1 Hz, 2H, CH$_2$C<u>H</u>$_2$CO), 2.13 (p, *J* = 6.6 Hz, 2H, CH$_2$C<u>H</u>$_2$CH$_2$), 1.24 (t, *J* = 7.1 Hz, 3H, CH$_2$C<u>H</u>$_3$).

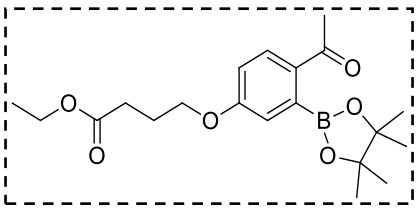
^{13}C NMR (101 MHz, $CDCl_3$): δ 195.14 (s, C<u>C</u>OCH$_3$), 172.93 (s, CH$_2$<u>C</u>OO), 162.96 (s, (CH)$_2$<u>C</u>$_{arom}$OCH$_2$), 148.58 (s, <u>C</u>$_{arom}$OTfC), 132.89 (s, <u>C</u>H$_{arom}$), 124.05 (s, <u>C</u>$_{arom}$COCH$_3$), 118.70 (q, *J* = 320.6 Hz, -SO$_2$<u>C</u>F$_3$), 113.68 (s, <u>C</u>H$_{arom}$), 109.53 (s, <u>C</u>H$_{arom}$), 67.85 (s, O<u>C</u>H$_2$CH$_2$), 60.71 (s, O<u>C</u>H$_2$CH$_3$), 30.49 (s, CH$_2$<u>C</u>H$_2$CO), 29.15 (s, CO<u>C</u>H$_3$), 24.27 (s, CH$_2$<u>C</u>H$_2$CH$_2$), 14.28 (s, CH$_2$<u>C</u>H$_3$).

HRMS (ESI): *m/z* calculated $[M+K]^+$ = 437.027867, found $[M+K]^+$ = 437.027219.

- Synthesis of compound **142**

Literature basis - Patent: Anacor Pharmaceuticals, Inc.; GlaxoSmithKline; US2010/256092; (**2010**); (A1) English

In a flame dried round bottom flask, under inert atmosphere, **144** (0.5 g, 1.25 mmol), bis(pinacolato)diboron (**120**, 0.6 g, 2.51 mmol), [1,1'-bis(diphenylphosphino)ferrocene]palladium (II) chloride (0.1 g; 0.125 mmol) and sodium acetate (0.3 g, 3.76 mmol) were flushed 3 times with Argon. Then, 8 mL of previously distilled dioxane were added and degassed for 10 minutes with bubbling Argon. The reaction mixture was heated at 95 ºC for 4 h (third reaction of Scheme **53**, *p.93*), after which the solvent was concentrated in vacuum and the crude residue purified by flash column chromatography (silica gel, dichloromethane:diethyl ether (19:1 – 9:1)) to give **142** as a white solid (0.3 g, 64%).



^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (d, *J* = 8.6 Hz, 1H, C<u>H</u>$_{arom}$), 6.93 (s, 1H, C<u>H</u>$_{arom}$), 6.82 (d, *J* = 8.6 Hz, 1H, C<u>H</u>$_{arom}$), 4.11 (q, *J* = 7.1 Hz, 2H, OC<u>H</u>$_2$CH$_3$), 4.04 (t, *J* = 6.0 Hz, 2H, OC<u>H</u>$_2$CH$_2$), 2.54 – 2.45 (m, 5H, COC<u>H</u>$_3$, CH$_2$C<u>H</u>$_2$CO), 2.12 – 2.05 (m, 2H, CH$_2$C<u>H</u>$_2$CH$_2$), 1.41 (s, 12H, 2xC(C<u>H</u>$_3$)$_2$), 1.23 (t, *J* = 7.1 Hz, 3H, OCH$_2$C<u>H</u>$_3$).

^{13}C NMR (101 MHz, $CDCl_3$): δ 198.54 (s, C$_{arom}$<u>C</u>OCH$_3$), 173.16 (s, CH$_2$<u>C</u>OO), 162.57 (s, <u>C</u>$_{arom}$OCH$_2$), 133.47 (s, CH$_3$CO<u>C</u>$_{arom}$), 130.79 (s, <u>C</u>H$_{arom}$), 117.94 (s, <u>C</u>H$_{arom}$), 113.98 (s, <u>C</u>H$_{arom}$), 83.63 (s, <u>C</u>(CH$_3$)$_2$), 66.87 (s, O<u>C</u>H$_2$CH$_2$), 60.54 (s O<u>C</u>H$_2$CH$_3$), 30.60 (s, CH$_2$<u>C</u>H$_2$CO), 24,95 (s, C(<u>C</u>H$_3$)$_2$), 24.54 (s, <u>C</u>H$_3$COC$_{arom}$), 24.47 (s, CH$_2$<u>C</u>H$_2$CH$_2$), 14.32 (s, OCH$_2$<u>C</u>H$_3$).

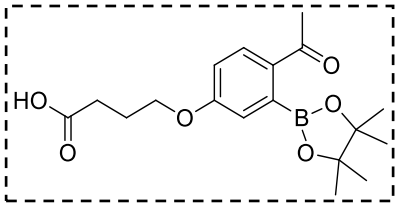
^{13}C-HMBC 138.5 (s, <u>C</u>$_{arom}$B)

HRMS (ESI): *m/z* calculated $[M+H]^+$ = 377.213364, found $[M+H]^+$ = 377.211988.

- Synthesis of compound **141**

Literature basis - Griffin, D.R.; Andrea M. Kasko, A.M.; *Journal of the American Chemical Society* **2012**, *134*, 13103

In a round bottom flask, under inert atmosphere, compound **142** (1.3 g, 3.4 mmol) was stirred in a solution of trifluoroacetic acid (1 mL) and water (9.5 mL) at 90 ºC for 3 h (fourth reaction of Scheme **53**, *p.93*), after which the solvent was concentrated in vacuo. The product was purified by flash column chromatography (silica gel, dichloromethane:diethyl ether 9:1 – 1:1) and recrystallized in diethyl ether to afford **141** (0.9 g, 77%).



^{1}H NMR (400 MHz, $CDCl_3$): δ 7.77 (d, *J* = 8.6 Hz, 1H, C$\underline{H}_{arom}$), 6.96 (d, *J* = 2.3 Hz, 1H, C$\underline{H}_{arom}$), 6.85 (dd, *J* = 8.6, 2.3 Hz, 1H, C$\underline{H}_{arom}$), 4.09 (t, *J* = 6.0 Hz, 2H, OC$\underline{H}_2$CH$_2$), 2.58 (t, *J* = 7.1 Hz,2H, CH$_2$C$\underline{H}_2$COOH), 2.55 (s, 3H, COC$\underline{H}_3$), 2.12 – 2.05 (m, 2H, CH$_2$C$\underline{H}_2$CH$_2$), 1.44 (s, 12H, 2xC(C$\underline{H}_3$)$_2$).

^{13}C NMR (101 MHz, $CDCl_3$): δ 198.92 (s, C$_{arom}\underline{C}$OCH$_3$), 178.49 (s, CH$_2\underline{C}$OO), 162.64 (s, $\underline{C}_{arom}$OCH$_2$), 133.53 (s, CH$_3$CO$\underline{C}_{arom}$), 130.83 (s, $\underline{C}$H$_{arom}$), 117.86 (s, $\underline{C}$H$_{arom}$), 114.16 (s, $\underline{C}$H$_{arom}$), 83.73 (s, $\underline{C}$(CH$_3$)$_2$), 66.75 (s, O$\underline{C}$H$_2$CH$_2$), 30.39 (s, CH$_2\underline{C}$H$_2$CO), 24.99 (s, C($\underline{C}$H$_3$)$_2$), 24.77 (s, CO$\underline{C}$H$_3$), 24.28 (s, CH$_2\underline{C}$H$_2$CH$_2$).

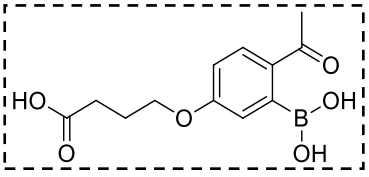
^{13}C-HMBC 138.7 (s, $\underline{C}_{arom}$B)

HRMS (ESI): *m/z* calculated $[M+H]^+$ = 349.182029, found $[M+H]^+$ = 349.181109.

- Synthesis of compound **140**

Literature basis - Pennington, T.E.; Kardiman, C.; Hutton, C.A.; *Tetrahedron Letters* **2004**, *45*, 6657

Treatment of compound **141** with 5 equiv of polystyrene–boronic acid (**146**) in acetonitrile/1 M HCl (9:1) for 20 h at 25 ºC (fifth reaction of Scheme **53**, *p.93*) gave 8% yield of compound **140**, after evaporating solvents and performing a flash column chromatography (silica gel, acetone:methanol).



^{1}H NMR (400 MHz, D_2O) δ 7.95 (d, *J* = 8.0 Hz, 1H, C$\underline{H}_{arom}$), 6.96 (s, 2H, 2xC$\underline{H}_{arom}$), 4.13-4.09 (m, 2H, OC$\underline{H}_2$CH$_2$), 2.55-2.47 (m, 5H, COC$\underline{H}_3$ and CH$_2$C$\underline{H}_2$CH$_2$), 2.03 (d, *J* = 7.0 Hz, 2H, -CH$_2$C$\underline{H}_2$COOH).

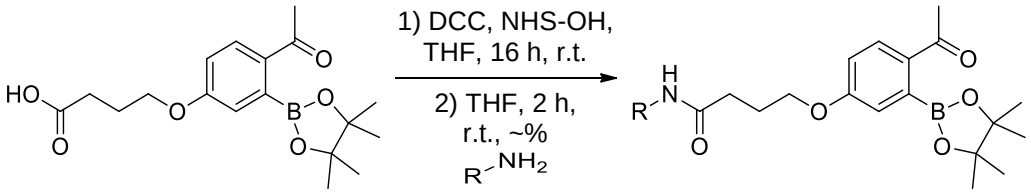
VI.4. Experimental Section of Chapter IV

VI.4.1. General Procedures

VI.4.1.1. Esterification or amidation of compound 141

- General Procedure for Amidation Reaction

Literature basis - Park, S. Y.; Oh, K. T.; Oh, Y. T.; Oh, N. M.; Youn, Y. S.; Lee, E. S. *Chemical Communication*, **2012**, *48*, 2522



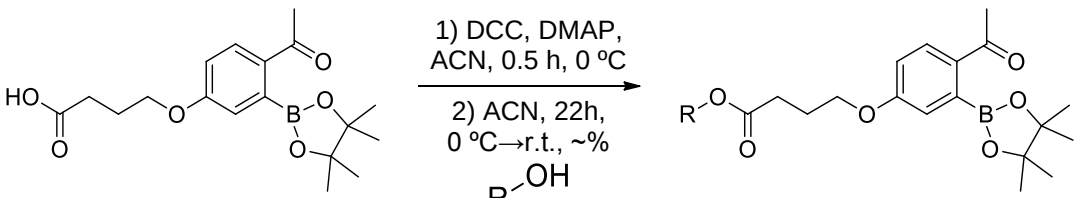
141 **218** **219**

Scheme **80** – General Procedure for Amidation of compound **141**

In a round bottom flask, under inert atmosphere, compound **141** (1 equivalent), N,N'-dicyclohexylcarbodiimide (DCC, 2 equivalents) and N-hydroxysuccinimide (NHS-OH, 1 equivalent) were mixed at room temperature in previously distilled tetrahydrofuran (0.06 M) and stirred overnight. Then, DCU was filtered from the reaction and the amine derivative (**218**x, 1.2 equivalents) was added and the solution stirred for 2 h at room temperature (Scheme **80**). After that, the solvent was concentrated under reduced pressure and redissolved in ethyl acetate, which was then washed with water and brine. The solvents were evaporated under reduced pressure and the crude products were further purified by flash column chromatography to afford different compounds with different yields (**219**x).

- General Procedure for Esterification Reaction

Literature basis - Liu, Z.; Hu, J.; Sun, J.; He, G.; Li, Y.; Zhang, G.; *Journal of Polymer Science Part A: Polymer Chemistry* **2010**, *48 (16)*, 3573



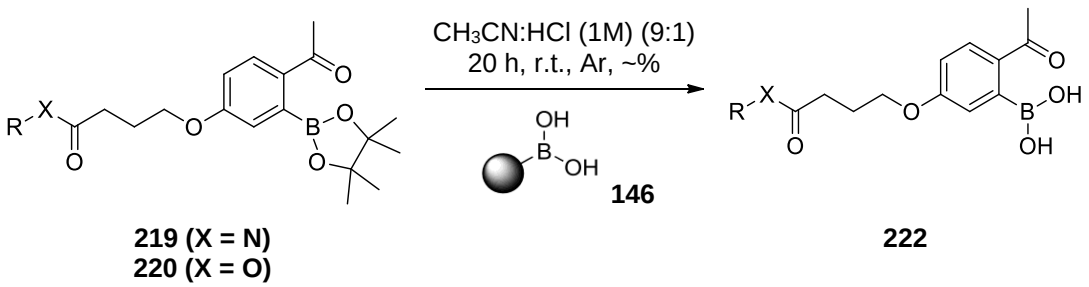
141 **221** **220**

Scheme **81** – General Procedure for Esterification of compound **141**

In a round bottom flask, under inert atmosphere, compound **141** (1 equivalent), N,N'-dicyclohexylcarbodiimide (DCC, 3 equivalents) and 4-dimethylaminopyridine (DMAP, 0.1 equivalent) were mixed at 0 ºC in previously distilled acetonitrile (ACN, 0.11 M) and stirred for 30 minutes. A solution of the alcohol derivative (**221**x, 2 equivalents) in previously distilled acetonitrile (0.22 M) was added *via* a syringe and the solution was stirred for 22 h at room temperature (Scheme **81**). The solvent was, then, concentrated *in vacuo* and the residues were purified by flash column chromatography to afford different compounds with different yields (**220**x).

VI.4.1.2. Deprotection of pinacolyl boronate ester

Literature basis - Pennington, T.E.; Kardiman, C.; Hutton, C.A.; *Tetrahedron Letters* **2004**, *45*, 6657



Scheme **82** – General procedure for deprotection of pinacolyl boronate ester of compounds **219x** and **220x**

Treatment of compound **219**x or **220**x with 5 equiv of polystyrene–boronic acid in acetonitrile/1 M HCl (9:1) for 20 h at 25 ºC (Scheme **82**), gave different yields of compounds **222**x, after evaporating solvents and performing a flash column chromatography. These compounds were immediately used for conjugation after deprotection.

VI.4.2. Experimental Details of Glycosilation

VI.4.2.1. Direct insertion of glycosyl derivative

- First Attempt to synthesize a glycosyl derivative **148** (Unsuccessful)

Based on the general procedure for esterification of compound **141**, using freshly distilled dichloromethane instead of THF and D-glucose (**147**) as the alcohol derivative (Scheme **54**, *p.102*), an

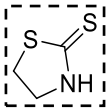
attempt was made to synthesize compound **148** though after a preparative chromatography (silica gel, ethyl ether) no product with aromatic proton signals was ever detected.

- Second Attempt to synthesize a glycosyl derivative **148**

Step 1 – Synthesis of compound **131**

Literature Basis - Chen, N.; Jia, W.; Xu, J. *European Journal of Organic Chemistry* **2009** (*33),* 5841

In a round bottom flask, 2-ethanolamine (**150**, 0.6 mL, 0.01 mol) was dissolved in an aqueous solution of potassium hydroxide (50 mL of KOH (1 M)) and, then, carbon disulfide (**149**, 3.1 mL, 0.05 mol) was added. The reaction mixture was heated at reflux for 16 h (top reaction of Scheme **55**, *p.103*). After cooling to room temperature, the reaction mixture was extracted with dichloromethane (3x20 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure to give the crude product, which was purified by recrystallization with ethyl acetate to afford the product **131** as colorless crystals (0.4 g, 35%).



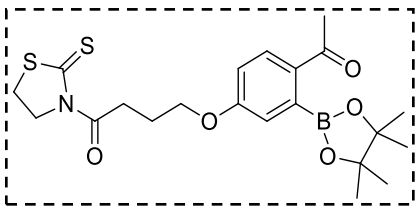
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (s, 1H, $CH_2N\underline{H}CS$), 3.99 (t, *J* = 7.6 Hz, 2H, -$CH_2\underline{C}H_2NH$-), 3.55 (t, *J* = 7.6 Hz, 2H, -$S\underline{C}H_2CH_2$-).

^{13}C NMR (101 MHz, $CDCl_3$) δ 201.84 (s, -$S\underline{C}SNH$-), 51.46 (s, -$CH_2\underline{C}H_2NH$-), 33.77 (s, -$S\underline{C}H_2CH_2$-).

Step 2 – Synthesis of compound **152**

Literature Basis - Baczko, K.; Plusquellec, D. *Tetrahedron Letters* **1991**, 47(23), 3817

In a round bottom flask, under inert atmosphere, compound **141** (0.1 g, 0.3 mmol) and N,N'-dicyclohexylcarbodiimide (DCC, 0.09 g, 0.43 mmol) were dissolved in 5 mL of distilled ethyl acetate. Then, compound **130** (34 mg, 0.3 mmol) was added and the compounds were left reacting for 40 h at room temperature (middle reaction from Scheme **55**, *p.103*). After that, the reaction mixture was filtrated and the solution concentrated under reduced pressure. The crude product was further purified flash column chromatography (silica gel, dichloromethane:diethyl ether (1:0 – 1:1)) to afford compound **152** in 31% yield (40 mg).



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 8.6 Hz, 1H, $C\underline{H}_{arom}$), 6.95 (d, *J* = 2.0 Hz, 1H, $C\underline{H}_{arom}$), 6.86 (dd, *J* = 8.6, 2.0 Hz, 1H, $C\underline{H}_{arom}$), 4.57 (t, *J* = 7.5 Hz, 2H, $NC\underline{H}_2CH_2S$), 4.09 (t, *J* = 6.0 Hz, 2H, $OC\underline{H}_2CH_2$), 3.44 (t, *J* = 6.9 Hz, 2H, $CH_2C\underline{H}_2CON$), 3.27 (t, *J*

= 7.5 Hz, 2H, NCH_2CH_2S), 2.54 (s, 3H, $COCH_3$), 2.18 (p, *J* = 6.4 Hz, 2H, $CH_2CH_2CH_2$), 1.43 (s, 12H, $2xC(CH_3)_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 201.74 (s, NCSS), 198.57 (s, $C_{arom}COCH_3$), 174.11 (s, CH_2CON), 162.62, 133.54 (s, CH_3COC_{arom}), 130.87 (s, CH_{arom}), 117.96 (s, CH_{arom}), 114.19 (s, CH_{arom}), 83.70 (s, $C(CH_3)_2$), 66.81 (s, OCH_2CH_2), 56.05 (s, $CONCH_2CH_2$), 35.12 (s, NCH_2CH_2S), 28.40 (s, $CH_2CH_2CH_2$), 25.04 (s, $C(CH_3)_2$), 24.93 (s, $COCH_3$), 24.37 (s, CH_2CH_2CO).

^{13}C-HMBC 138.5 (s, $C_{arom}B$)^

Step 3 – Glycosyl derivative selective esterification (Unsuccessful)

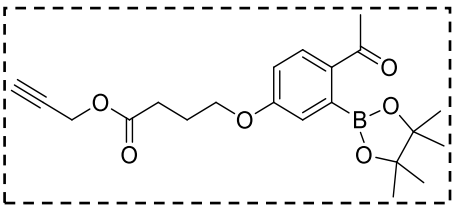
Literature Basis - Baczko, K.; Plusquellec, D. *Tetrahedron Letters* **1991**, 47(23), 3817

In a round bottom flask, under inert atmosphere, compound **152** (40 mg, 0.09 mmol) was dissolved in 0.5 mL of freshly distilled pyridine (over CaH_2). Then, 4-dimethylaminopyridine (DMAP, 9 mg, 0.07 mmol) and sodium hydride (60%, 2 mg, 0.04 mmol) were added, followed by D-glucose (35 mg, 0.18 mmol). Compounds were left reacting at room temperature for 24 h (last reaction of Scheme **55**, *p.103*), after which, acetic acid was added to the reaction mixture and the reaction mixture concentrated under reduced pressure. Multiple extractions between different aqueous and organic phases were made, though the evaluation of the crude product never indicated the presence of compound **148**.

VI.4.2.2. Indirect insertion of glycosyl derivative

- Synthesis of pinacolyl boronate ester of compound **154** (**222**a)

Following the general procedure for esterification of compound **141**, using propargyl alcohol as the alcohol derivative (0.6 mmol), EDC instead of DCC and DCM instead of ACN, after 17 h, compound **222**a was obtained as a white solid in 74% yield (0.1 g).



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 8.6 Hz, 1H, CH_{arom}), 6.95 (d, *J* = 1.9 Hz, 1H, CH_{arom}), 6.84 (dd, *J* = 8.6, 2.0 Hz, 1H, CH_{arom}), 4.69 (d, *J* = 1.8 Hz, 2H, $-OCH_2CCH$), 4.07 (t, *J* = 6.0 Hz, 2H, $-OCH_2CH_2-$), 2.60 – 2.53 (m, 5H, $-CH_2CH_2COO-$ and $C_{arom}COCH_3$), 2.48 (s, 1H, $-OCH_2CCH$), 2.17 – 2.10 (m, 2H, $-CH_2CH_2CH_2-$), 1.43 (s, 12H, $2xC(CH_3)_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 198.59 (s, $C_{arom}COCH_3$), 172.37 (s, $-CH_2COO-$), 162.56 (s, $C_{arom}OCH_2$), 133.64 (s, $C_{arom}COCH_3$), 130.86 (s, CH_{arom}), 117.94 (s, CH_{arom}), 114.17 (s, CH_{arom}), 83.79 (s, $C(CH_3)_2$),

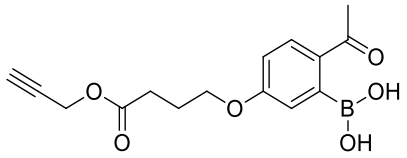
77.69 (s, $CH_2\underline{C}CH$), 75.11 (s, -$OCH_2\underline{C}\underline{C}H$), 66.74 (s, -$O\underline{C}H_2CH_2$-), 52.13 (s, -$O\underline{C}H_2CCH$), 30.42 (s, $CH_2\underline{C}H_2CO$), 25.07 (s, -$C(\underline{C}H_3)_2$), 24.96 (s, $C_{arom}CO\underline{C}H_3$), 24.45 (s, -$CH_2\underline{C}H_2CH_2$-).

^{13}C-HMBC 138.6 (s, $\underline{C}_{arom}B$)

HRMS (ESI): *m/z* calculated $[M+H]^+$ = 387.19773, found $[M+H]^+$ = 387.19765.

- Synthesis of compound **154**

Following the general procedure of deprotection of pinacolyl boronate ester and using compound **222**a as the starting material (64 mg, 0.2 mmol), after 25 h, compound **154** was obtained as a white solid in 61% yield (34 mg).



HRMS (ESI): *m/z* calculated $[M+H]^+$ = 327.10132, found $[M+H]^+$ = 327.10152.

- Conjugation of compound **154** with Lysozyme

Compound **154** dissolved in DMSO (1 mM) reacted with 10 μM of lysozyme in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes of reaction the solution was evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. HRMS results of deconvulted masses for unconjugated and conjugated species are shown in Table **22**.

Table **22** – Conjugation of compound **154** with Lysozyme

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Lysozyme	14296.84832	$[M+H]^+$	52.1	-
Lysozyme + ***154***	14582.95071	$[M+H]^+$	100.0	14582.94930
Lysozyme + *2x**154***	14869.08390	$[M+H]^+$	27.8	14869.05029
Lysozyme + *3x**154***	15154.15850	$[M+H]^+$	46.8	15155.15127
Lysozyme + *4x**154***	15441.27006	$[M+H]^+$	34.6	15441.252260
Lysozyme + *5x**154***	15727.38332	$[M+H]^+$	15.5	15727.353245
Lysozyme + *6x**154***	16012.47857	$[M+H]^+$	15.2	16012.44641

- CuAAC on Lysozyme's surface

Based on the procedure to conjugate compound **154** with lysozyme in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature, an ESI-FTICR-MS and the conjugated species were detected. Afterwards, compound **155** dissolved in DMSO (5 mM) was added to the conjugated lyzosyme, alongside with copper sulfate (200 µM) and sodium ascorbate (5 mM) and this mixture was left reacting overnight at 37 ºC and 1200 rpm. After that, an ESI-FTICR-MS was performed to detect the result of a click reaction. HRMS results of click product, as well as the deconvulted masses for unconjugated and conjugated species are shown in Table **23**.

Table **23** – CuAAC reaction between compound **154** and **155** on Lysozyme's surface

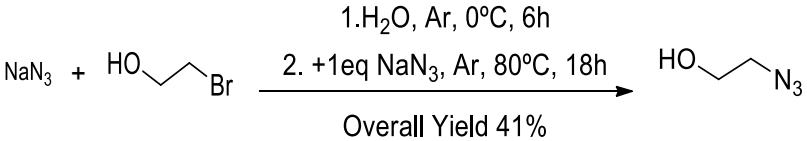
Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
154-155	696.20458	$[M+H]^+$	-	696.20104
Lysozyme	14297.82149	$[M+H]^+$	100.0	-
Lysozyme + ***154-155***	14953.08122	$[M+H]^+$	0.48	14953.02274
Lysozyme + ***154-155***	14934.09105	$[M+H-H_2O]^+$	1.11	14934.01218
Lysozyme + ***154-155***	14918.22193	$[M+H-2H_2O]^+$	0.15	14918.00161

VI.4.3. Experimental Details of Bioimaging

VI.4.3.1. Indirect attachment of a fluorescent probe

- Synthesis of compound **156**

Literature basis – Wu, J.-C.;Wang, D.-X.;Huang, Z.-T.; Wang, M-X *Journal of Organic Chemistry,* **2010**, *75(24)*, 8604

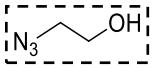


223 **224** **156**

Scheme **83** – Synthesis of compound **156**

In a round bottom flask, sodium azide (**223**, 1.0 g, 16.0 mmol) was slowly added to a solution of 2-bromoethanol (**224**, 0.6 mL, 8.0 mmol) in 1 mL of water that was stirring over an ice bath. Then, the reactions were left stirring until they reach the room temperature. After 6 h, another equivalent of sodium

azide (**223**, 0.5 g, 8.0 mmol) was added and the reaction mixture heated at 80 ºC over 18 h (Scheme **83**). After that time, the reaction was cooled down to room temperature and the residue was extracted with diethyl ether (10x10 mL), dried with $MgSO_4$, filtered and the solvent evaporated to afford 2-azidoethanol (**156**) as a colorless oil (0.3 g, 41%).



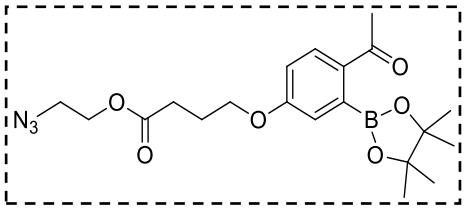
^{1}H NMR (400 MHz, $CDCl_3$) δ 3.68 (d, *J* = 3.0 Hz, 2H, $CH_2C\underline{H_2}OH$), 3.34 (d, *J* = 2.0 Hz, 2H, $N_3C\underline{H_2}CH_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 61.07 (s, $CH_2\underline{C}H_2OH$), 53.23 (s, $N_3\underline{C}H_2CH_2$).

NMR characterization in accordance with the one described in literature

- Synthesis of pinacolyl boronate ester of **157** (**222**b)

Following the general procedure for esterification of compound **141**, using 2-azidoethanol (**156**) as the alcohol derivative (0.6 mmol), EDC instead of DCC and DCM instead of ACN, after 24 h, compound **222**b was obtained as a white solid in 75% yield (0.09 g).



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 8.6 Hz, 1H, $C\underline{H}_{arom}$), 6.95 (d, *J* = 1.2 Hz, 1H, $C\underline{H}_{arom}$), 6.84 (dd, *J* = 8.6, 1.2 Hz,1H, $C\underline{H}_{arom}$), 4.28 – 4.23 (m, 2H, $-OC\underline{H_2}CH_2N_3$), 4.08 (t, *J* = 5.9 Hz, 2H, $-OC\underline{H_2}CH_2CH_2-$), 3.49 – 3.43 (m, 2H, $-OCH_2C\underline{H_2}N_3$), 2.61 – 2.52 (m, 5H, $-CH_2\underline{C}H_2COO-$ and $C_{arom}COC\underline{H_3}$), 2.13 (p, *J* = 6.5 Hz, 2H, $-CH_2C\underline{H_2}CH_2-$), 1.43 (s, 12H, $2xC(C\underline{H_3})_2$).

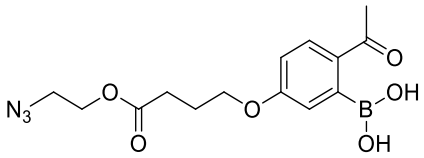
^{13}C NMR (101 MHz, $CDCl_3$) δ 198.48 (s, $C_{arom}\underline{C}OCH_3$), 172.79 (s, $-CH_2\underline{C}OO-$), 162.55 (s, $\underline{C}_{arom}OCH_2$), 133.69 (s, $C_{arom}\underline{C}OCH_3$), 130.83 (s, $\underline{C}H_{arom}$), 118.01 (s, $\underline{C}H_{arom}$), 114.12 (s, $\underline{C}H_{arom}$), 83.71 (s, $\underline{C}(CH_3)_2$), 66.82 (s, $-O\underline{C}H_2CH_2-$), 63.13 (s, $-O\underline{C}H_2CH_2N_3$), 49.83 (s, $-OCH_2\underline{C}H_2N_3$), 30.50 (s, $-CH_2\underline{C}H_2CO-$), 25.05 (s, $-C(\underline{C}H_3)_2$), 24.93 (s, $C_{arom}CO\underline{C}H_3$), 24.46 (s, $-CH_2\underline{C}H_2CH_2-$).

^{13}C-HMBC 138.6 (s, $\underline{C}_{arom}B$)

HRMS (ESI): *m/z* calculated $[M+H]^+$ = 418.21476, found $[M+H]^+$ = 418.21457.

- Synthesis of compound **157**

Following the general procedure of deprotection of pinacolyl boronate ester and using compound **222**b as the starting material (68 mg, 0.2 mmol), after 20 h, compound **157** was obtained as a white solid in 68% yield (37 mg).



HRMS (ESI): *m/z* calculated $[M+Na]^+$ = 358.11835, found $[M+Na]^+$ = 358.11896.

- Conjugation of compound **157** with Lysozyme

Compound **157** dissolved in DMSO (1 mM) reacted with 10 μM of lysozyme in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes of reaction the solution was evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. HRMS results of deconvulted masses for unconjugated and conjugated species are shown in Table **24**.

Table **24** – Conjugation of compound **157** with Lysozyme

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Lysozyme	14296.81535	$[M+H]^+$	51.7	-
Lysozyme + ***157***	14614.03345	$[M+H]^+$	100.0	14614.04070
Lysozyme + *2x**157***	14931.15101	$[M+H]^+$	86.7	14931.15926
Lysozyme + *3x**157***	15248.30297	$[M+H]^+$	42.9	15248.27783
Lysozyme + *4x**157***	15565.40485	$[M+H]^+$	50.3	15565.39639
Lysozyme + *5x**157***	15881.52008	$[M+H]^+$	37.5	15881.50740
Lysozyme + *6x**157***	16198.65607	$[M+H]^+$	14.2	16198.68338

- SPAAC on Lysozyme's surface

Based on the procedure to conjugate compound **157** with lysozyme in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature, an ESI-FTICR-MS and the conjugated species were detected. Afterwards, compound **158** dissolved in DMSO (5 mM) was added to the conjugated lyzosyme and, after 1 h, an ESI-FTICR-MS was performed to detect the result of a click reaction. HRMS results of deconvulted masses for unconjugated and conjugated species are shown in Table **25**.

Table **25** - SPAAC reaction between compound **157** and **158** on Lysozyme's surface

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Lysozyme	14299.76846	$[M+H]^+$	97.6	-
Lysozyme + ***157-158***	15330.25383	$[M+H]^+$	0.4	15330.22730

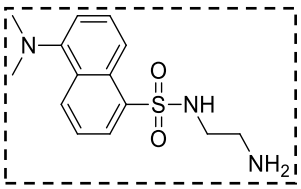
VI.4.3.2. Direct attachment of a fluorescent probe

VI.4.3.2.1. *Cell internalization study*

- Synthesis of 2-Dansylaminoethylamine ($DNH(CH_2)_2NH_2$, **162**)

Literature basis - Sun, W.; Bandmann, H., Schrader, T.; *Chemistry - A European Journal* **2007**, *13*, 7701

In a round bottom flask, 1,2-ethylenediamine (**161**, 7.4 mL, 111 mmol) was diluted in dichloromethane previously distilled over CaH_2 (25 mL) and cooled to 0 ºC, while stirring. A solution of dansyl chloride (**159**, 1.0 g, 3.7 mmol) in dichloromethane 16 mL was added dropwise. The mixture was stirred while warming to room temperature (reaction on the left of Scheme **58**, *p.106*). It was subsequently acidified with HCl (1 M) and then extracted with dichloromethane (3x20 mL). The organic layer was dried ($MgSO_4$) and concentrated to afford $DNH(CH_2)_2NH_2$ as a light yellow solid (1.02 g, 94%).



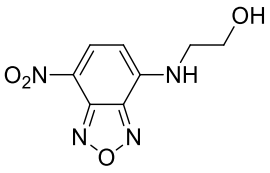
^{1}H NMR (400 MHz, CDCl3) δ 8.53 (d, *J* = 8.5 Hz, 1H, C<u>H</u>$_{arom}$), 8.31 (d, *J* = 8.5 Hz, 1H, C<u>H</u>$_{arom}$), 8.24 (d, *J* = 7.5 Hz, 1H, C<u>H</u>$_{arom}$), 7.57 – 7.49 (m, 2H, 2xC<u>H</u>$_{arom}$), 7.17 (d, J = 7.5 Hz, 1H, C<u>H</u>$_{arom}$), 2.93 – 2.86 (m, 8H, $C_{arom}N(C\underline{H}_3)_2$ and -NHC<u>H</u>$_2$C<u>H</u>$_2$NH$_2$), 2.69 (t, J = 5.7 Hz, 2H, -NHCH$_2$C<u>H</u>$_2$NH$_2$).

^{13}C NMR (101 MHz, CDCl3) δ 152.11 (s, $C_{arom}N(CH_3)_2$), 134.88 (s, <u>C</u>$_{arom}$SO$_2$NH), 130.50 (s, <u>C</u>H$_{arom}$), 130.01 (s, <u>C</u>H$_{arom}$), 129.73 (s, CN<u>C</u>$_{arom}$CH), 129.68 (s, <u>C</u>H$_{arom}$), 128.48 (s, CH<u>C</u>$_{arom}$CSO$_2$), 123.30 (s, <u>C</u>H$_{arom}$), 118.86 (s, <u>C</u>H$_{arom}$), 115.31 (s, <u>C</u>H$_{arom}$), 45.62 (s, -NH<u>C</u>H$_2$CH$_2$NH$_2$) 45.52 (s, $C_{arom}N(\underline{C}H_3)_2$), 40.99 (s, -NHCH$_2$<u>C</u>H$_2NH_2$).

NMR characterization in accordance with the one described in literature

- Synthesis of 4-(2-Hydroxyethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-NH(CH$_2$)$_2$OH, **163**)

Literature basis - Onoda, M.; Uchiyama, S., Santa, T., Imai, K.; *Analytical Chemistry* **2002**, *74(16)*, 4089



In a round bottom flask, under inert atmosphere, 4-Chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl, **160**, 100 mg, 0.50 mmol) was dissolved in acetonitrile (10 mL). After the addition of 2-ethanolamine (**150**, 200 µL) in acetonitrile (5 mL), the solution was stirred at room temperature for 30 minutes (reaction on the right of Scheme **58**, *p.106*). The reaction mixture was concentrated and the residue was purified by column chromatography (silica gel, dichloromethane-methanol (1:0 – 19:1)) to afford NBD-NH$(CH_2)_2$OH as an orange solid in 49% yield. This compound was immediately used after being synthesized.

- Synthesis of N-(2-aminoethyl) folic acid (EDA-FA, **170**, Scheme **59**, *p.107*)

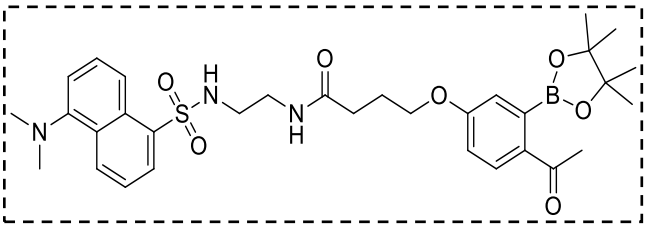
Literature basis - Rutnakornpituk, M.; Puangsin, N.; Theamdee, P.; Rutnakornpituk, B.; Wichai, U.; Polymer, **2011**, 52, 987

The derivative of folic acid (N-(2-aminoethyl) folic acid (EDA-FA)) was synthesized according to an existing procedure obtaining 69% in the first step and 92% in the second one.

HRMS (ESI): m/z calculated $[M+H]^+$ = 484.20514, found $[M+H]^+$ = 484.205640.

- Synthesis of compound **164**

Following the general procedure for amidation of compound **141**, using DNH$(CH_2)_2NH_2$ (**162**) as the amine derivative (0.34 mmol), after 2 h, compound **164** was obtained as a light yellow solid in 23% yield (41 mg).



^{1}H NMR (400 MHz, $CDCl_3$): δ 8.51 (d, *J* = 8.5 Hz, 1H, C$\underline{H}_{arom}$), 8.25 (d, *J* = 8.6 Hz, 1H, C$\underline{H}_{arom}$), 8.18 (d, *J* = 7.2 Hz, 1H, C$\underline{H}_{arom}$), 7.73 (d, *J* = 8.6 Hz, 1H, C$\underline{H}_{arom}$), 7.51 (dt, *J* = 19.6, 8.0 Hz, 2H, C$\underline{H}_{arom}$), 7.15 (d, *J* = 7.6 Hz, 1H, C$\underline{H}_{arom}$), 6.93 (d, *J* = 1.9 Hz, 1H, C$\underline{H}_{arom}$), 6.80 (dd, *J* = 8.6, 1.7 Hz, 1H, C$\underline{H}_{arom}$), 6.25 (s, 1H, CON$\underline{H}$CH$_2$), 6.00 (t, *J* = 5.6 Hz, 1H, CH$_2$N$\underline{H}$SO$_2$), 3.96 (t, *J* = 5.8 Hz, 2H, OC$\underline{H}_2$CH$_2$), 3.25 (dd, *J* = 10.3, 5.3 Hz, 2H, CONHC$\underline{H}_2$), 2.96 (dd, *J* = 10.3, 5.3 Hz, 2H, C$\underline{H}_2$NHSO$_2$), 2.85 (s, 6H, C$_{arom}$N(C$\underline{H}_3$)$_2$), 2.52 (s, 3H, COC$\underline{H}_3$), 2.17 (t, *J* = 7.2 Hz, 2H, CH$_2$C$\underline{H}_2$CONH), 2.00 – 1.96 (m, 2H, CH$_2$C$\underline{H}_2$CH$_2$), 1.42 (s, 12H, 2xC(C$\underline{H}_3$)$_2$).

^{13}C NMR (101 MHz, $CDCl_3$): δ 198.68 (s, C$_{arom}\underline{C}$OCH$_3$), 173.26 (s, CH$_2\underline{C}$ONH), 162.66 (s, $\underline{C}_{arom}$OCH$_2$), 152.11 (s, C$_{arom}$N(CH$_3$)$_2$), 134.45 (s, CH$\underline{C}_{arom}$SO$_2$), 133.50 (s, CH$_3$CO$\underline{C}_{arom}$), 130.95 (s, $\underline{C}H_{arom}$), 130.66 (s, $\underline{C}H_{arom}$), 129.95 (s, CN$\underline{C}_{arom}$CH), 129.67 (s, $\underline{C}H_{arom}$CSO$_2$), 129.54 (s, CHC$_{arom}$CSO$_2$), 128.60 (s, $\underline{C}H_{arom}$), 123.31 (s, $\underline{C}H_{arom}$), 118.73 (s, $\underline{C}H_{arom}$), 118.17 (s, $\underline{C}H_{arom}$), 115.33 (s, $\underline{C}H_{arom}$CNCH$_3$), 113.90 (s,

CH_{arom}), 83.72 (s, C$(CH_3)_2$), 67.09 (s, OCH_2CH_2), 45.49 (s, C_{arom}N(C$H_3)_2$), 43.25 (s, CH_2NHSO_2), 39.27 (s, CONHCH_2), 32.42 (s, CH_2CH_2CONH), 24,96 (s, C(C$H_3)_2$), 24,93 (s, CH_2CH_2CH_2), 24,87 (s, COCH_3).

^{13}C-HMBC 138.6 (s, C$_{arom}$B)

HRMS (ESI): *m/z* calculated $[M+K]^+$ = 662.247383, found $[M+K]^+$ = 662.247221.

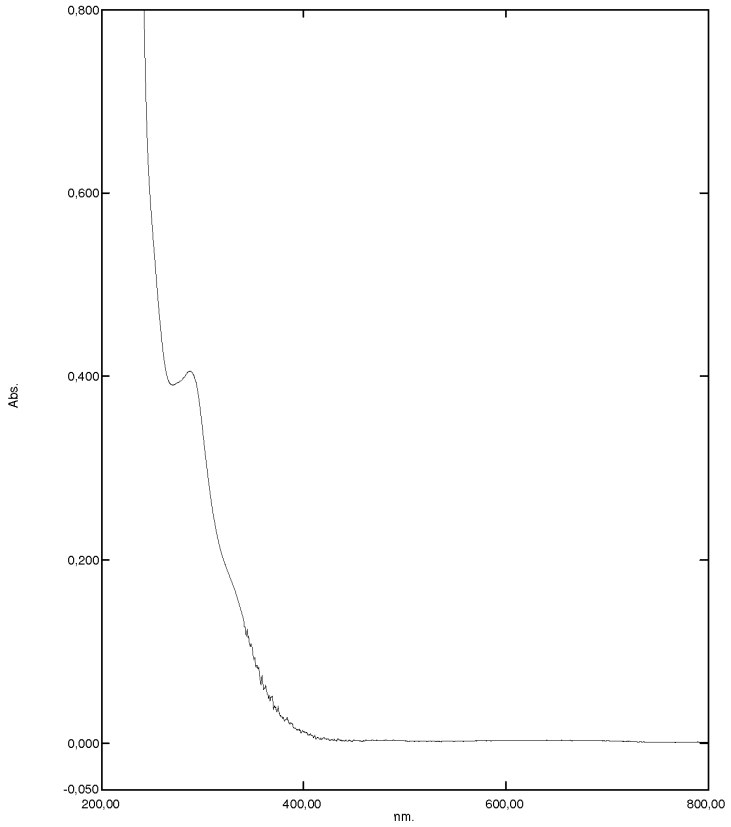


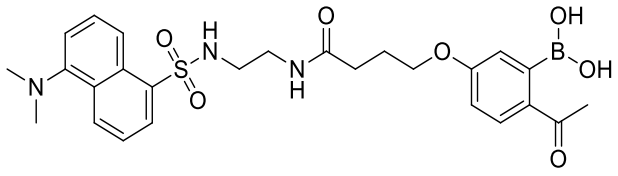
Figure **52** – UV spectrum of compound **164**

Applying the Beer–Lambert law:

λ_{max} = 290nm; Absorbance (Abs_{max})= 0.404; Molar absorption coefficient (ε)= 8080 $M^{-1}cm^{-1}$

- Synthesis of compound **171**

Following the general procedure of deprotection of pinacolyl boronate ester and using compound **164** as the starting material (14 mg, 0.02 mmol), after 18 h, compound **171** was obtained as a light green solid in 27% yield (3 mg).



- Conjugation of compound **171** with Lysozyme

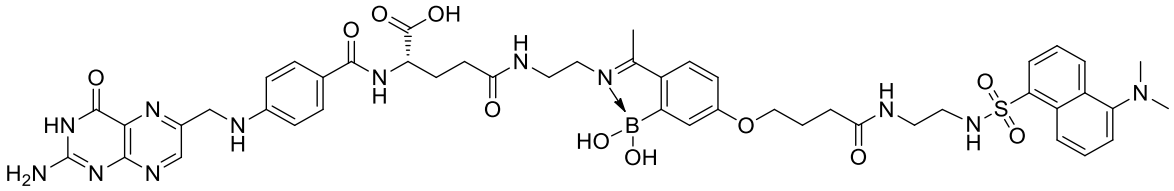
Compound **171** dissolved in DMSO (200 µM) reacted with 10 µM of lysozyme in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes of reaction the solution was evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. HRMS results of deconvulted masses for unconjugated and conjugated species are shown in Table **26**.

Table **26** – Conjugation of compound **171** with Lysozyme

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Lysozyme	14296.86852	$[M+H]^+$	100.0	-
Lysozyme + ***171***	14803.04136	$[M+H]^+$	28.6	14803.02332

- Conjugation of **171** with EDA-FA

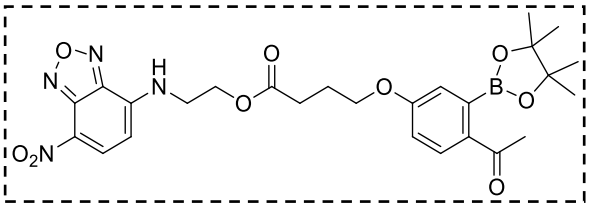
Compounds **171** was dissolved in DMSO, as well as EDA-FA. From these solutions compound **171** (100 µM) reacted with 100 µM of EDA-FA in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes, the reactions mixtures were evaluated by performing an ESI-FTICR-MS and the conjugated species were detected:



HRMS (ESI): *m/z* calculated $[M+H-H_2O]^+$ = 989.39022, found $[M+H-H_2O]^+$ = 989.39107

- Synthesis of compound **165**

Following the general procedure for esterification of compound **141**, using NBD-$NH(CH_2)_2OH$ (**163**) as the alcohol derivative (0.23 mmol), after 22 h, compound **165** was obtained as an orange solid in 88% yield (0.09 g).



^{1}H NMR (400 MHz, $CDCl_3$): δ 8.44 (d, *J* = 8.6 Hz, 1H, CHC<u>H</u>CNO_2), 7.71 (d, *J* = 8.6 Hz, 1H, C<u>H</u>*arom*), 6.90 (s, 1H, C<u>H</u>*arom*), 6.84 (s, 1H, CH_2N<u>H</u>C), 6.78 (d, *J* = 8.6 Hz, 1H, C<u>H</u>*arom*), 6.19 (d, *J* = 8.6 Hz, 1H, NHCC<u>H</u>CH), 4.45 (t, *J* = 5.0 Hz, 2H, OC<u>H_2</u>CH_2NH), 4.06 (t, *J* = 5.7 Hz, 2H, OC<u>H_2</u>CH_2), 3.75 (d, *J* = 5.0 Hz, 2H, OCH_2C<u>H_2</u>NH), 2.58 (t, *J* = 7.1 Hz, 2H, CH_2C<u>H_2</u>COO), 2.53 (s, 3H, COC<u>H_3</u>), 2.15 – 2.07 (m, 2H, CH_2C<u>H_2</u>CH_2), 1.42 (s, 12H, CC<u>H_3</u>).

^{13}C NMR (101 MHz, $CDCl_3$): δ 198.57 (s, C*arom*<u>C</u>OCH_3), 173.50 (s, CH_2<u>C</u>OO), 162.40 (s, <u>C</u>*arom*OCH_2), 144.32 (s, 2x<u>C</u>NO), 143.88 (s, C<u>C</u>NO_2CH), 143.67 (s, C<u>C</u>NHCH), 136.37 (s, CH<u>C</u>HCNO_2), 133.71 (s, CH_3CO<u>C</u>*arom*), 130.83 (s, <u>C</u>H*arom*), 118.04 (s, <u>C</u>H*arom*), 113.91 (s, <u>C</u>H*arom*), 99.05 (s, NHC<u>C</u>HCH), 83.78 (s, <u>C</u>$(CH_3)_2$), 66.69 (s, O<u>C</u>H_2CH_2), 61.85 (s, O<u>C</u>H_2CH_2NH), 43.21 (s, OCH_2<u>C</u>H_2NH), 30.52 (s, CH_2<u>C</u>H_2CO), 25.06 (s, C(<u>C</u>$H_3)_2$), 24.92 (s, CO<u>C</u>H_3), 24.41 (s, CH_2<u>C</u>H_2CH_2).

^{13}C-HMBC 138.5 (s, <u>C</u>*arom*B)

HRMS (ESI): *m/z* calculated $[M+Na]^+$ = 577.208100, found $[M+Na]^+$ = 577.207605.

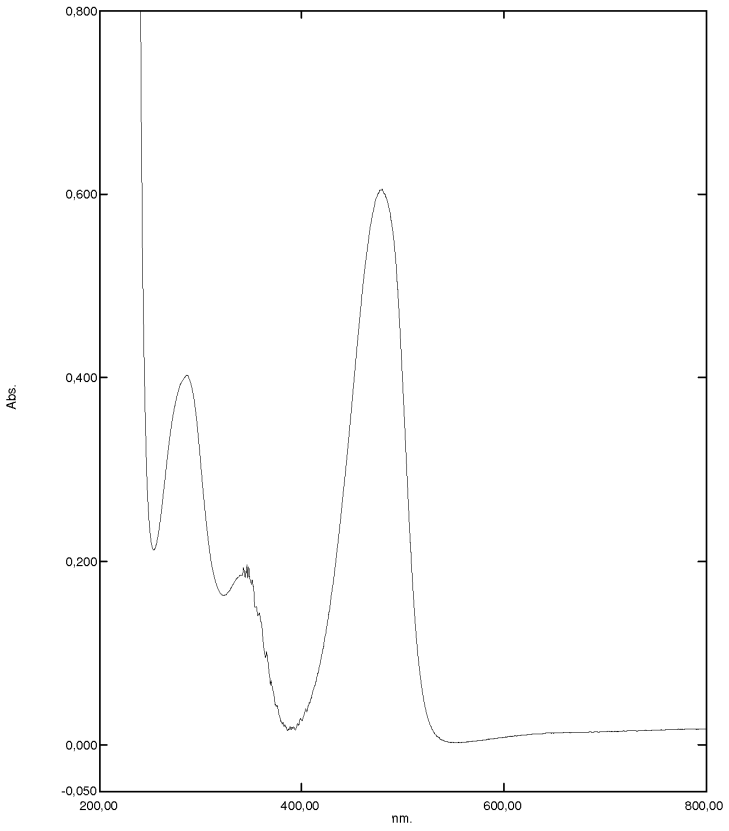


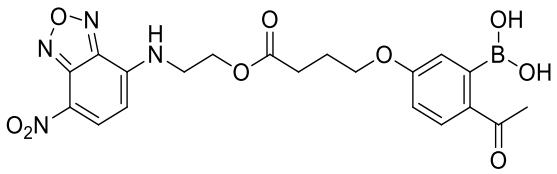
Figure **53** – UV spectrum of compound **165**

Applying the Beer–Lambert law:

λ_{max} = 480 nm; Absorbance (Abs_{max})= 0.604; Molar absorption coefficient (ε)= 12080 $M^{-1}cm^{-1}$

- Synthesis of compound **173**

Following the general procedure of deprotection of pinacolyl boronate ester and using compound **165** as the starting material (46 mg, 0.08 mmol), after 18 h, compound **173** was obtained as a orange solid in 27% yield (11 mg).



- Conjugation of compound **173** with Lysozyme

Compound **173** dissolved in DMSO (200 μM) reacted with 10 μM of lysozyme in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes of reaction the solution was evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. HRMS results of deconvulted masses for unconjugated and conjugated species are shown in Table **27**, Figure **54** and Figure **55**.

Table **27** – Conjugation of compound **173** with Lysozyme

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Lysozyme	14296.84145	$[M+H]^+$	100.0	-
Lysozyme + ***173***	14732.91497	$[M+H]^+$	15.1	14732.89023

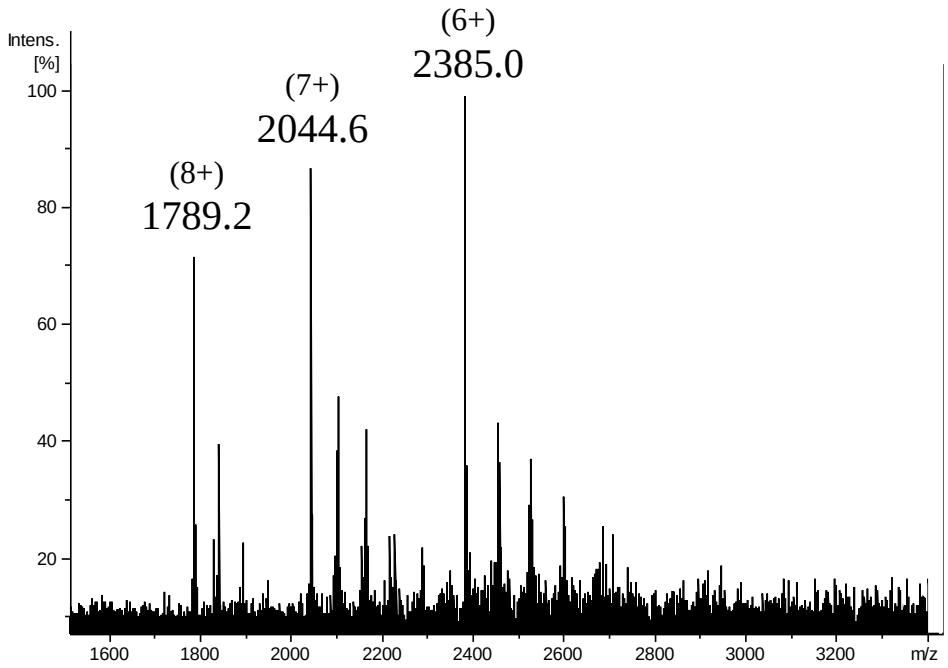


Figure **54** – Lyzosyme (10.0 µM) with compound **173** dissolved in DMSO (200.0 µM) in ammonium acetate buffer (50.0 mM, pH 7.0) after 30 minutes – Positive Mode of ESI-FTICR-MS full spectrum

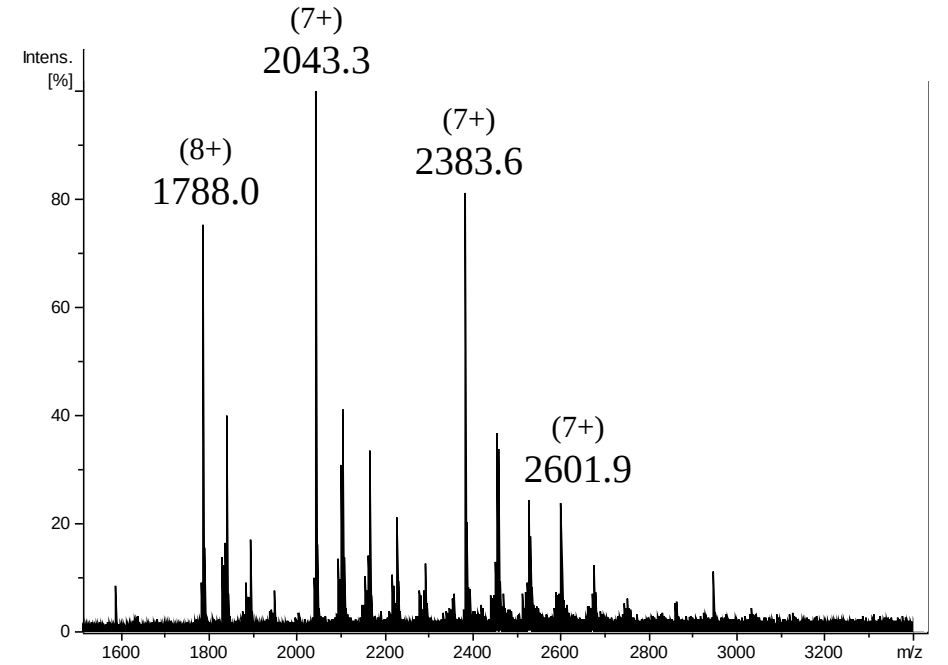
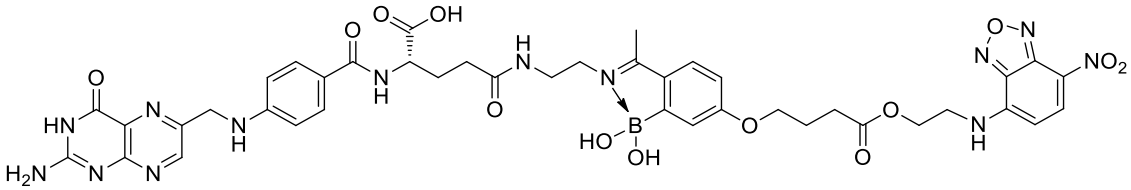


Figure **55** – Lyzosyme (10.0 µM) with compound **173** dissolved in DMSO (200.0 µM) in ammonium acetate buffer (50.0 mM, pH 7.0) after 2 h – Positive Mode of ESI-FTICR-MS full spectrum

- Conjugation of **173** with EDA-FA

Compounds **173** was dissolved in DMSO, as well as EDA-FA. From these solutions compounds **173** (100 µM) reacted with 100 µM of EDA-FA in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes, the reactions mixtures were evaluated by performing an ESI-FTICR-MS and the conjugated species were detected:

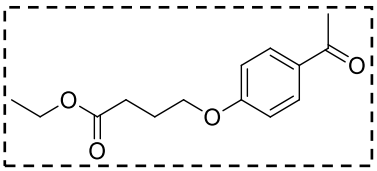


HRMS (ESI): *m/z* calculated $[M+H]^+$ = 938.33545, found $[M+H]^+$ = 938.33917

- Synthesis of compound **177**

Literature Basis – Al-Smadi, M.; Hanold, N.; Kalbitz, H.; Meier, H.; *Synthesis*, **2009**, *15*, 2539

In a round bottom flask (A), 4'-hydroxyacetophenone (**175**, 0.5 g, 3.6 mmol) and potassium carbonate (0.5 g, 3.6 mmol) were heated in acetone to reflux for 3 h. In a separate round bottom flask (B), ethyl 4-bromobutyrate (**176**, 0.8 mL, 5.5 mmol) and sodium iodide (0.8 g, 5.5 mmol) were stirred in acetone at room temperature for 2 h. Then, flask B was transferred to flask A and refluxed for 14 h (first reaction of Scheme **62**, *p.111*). After that, acetone was evaporated and 200 mL of water were added and extracted with diethyl ether (3x200 mL). The solution was dried (Na_2SO_4), concentrated and purified by column chromatography (silica gel, hexane:diethyl ether (9:1)) to give a viscous oil that solidified below 20 ºC; compound **177** (0.54 g, 59%).



^{1}H NMR (400 MHz, $CDCl_3$): δ 7.90 (d, *J* = 8.7 Hz, 2H, 2x$C\underline{H}_{arom}$), 6.90 (d, *J* = 8.7 Hz, 2H, 2x$C\underline{H}_{arom}$), 4.13 (q, *J* = 7.1 Hz, 2H, $OC\underline{H}_2CH_3$), 4.06 (t, *J* = 6.1 Hz, 2H, $OC\underline{H}_2CH_2$), 2.57 – 2.46 (m, 5H, $COC\underline{H}_3$, $COC\underline{H}_2CH_2$), 2.12 (p, *J* = 6.6 Hz, 2H, $CH_2C\underline{H}_2CH_2$), 1.24 (t, *J* = 7.1 Hz, 3H, $OCH_2C\underline{H}_3$).

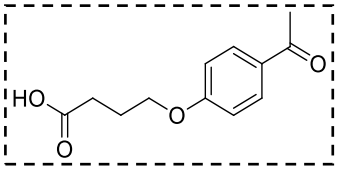
^{13}C NMR (101 MHz, $CDCl_3$): δ 196.84 (s, $C_{arom}\underline{C}OCH_3$), 173.11 (s, $O\underline{C}OCH_2$), 162.85 (s, $CH\underline{C}_{arom}OCH_2$), 130.67 (s, $\underline{C}H_{arom}$), 130.44 (s, $CH\underline{C}_{arom}COCH$), 114.22 (s, $\underline{C}H_{arom}$), 67.07 (s, $O\underline{C}H_2CH_2$), 60.60 (s, $O\underline{C}H_2CH_3$), 30.74 (s, $CO\underline{C}H_2CH_2$), 26.42 (s, $CO\underline{C}H_3$), 24.56 (s, $CH_2\underline{C}H_2CH_2$), 14.32 (s, $OCH_2\underline{C}H_3$).

HRMS (ESI): *m/z* calculated $[M+Na]^+$ = 273.109730, found $[M+Na]^+$ = 273.108601.

- Synthesis of compound **178**

Literature Basis – Griffin, D.R.; Andrea M. Kasko, A.M.; *Journal of the American Chemical Society* **2012**, *134*, 13103

In a round bottom flask, **177** (0.3 g, 1.2 mmol) was stirred in a solution of trifluoroacetic acid (TFA, 0.35 mL) and water (3.35 mL) at 90 ºC for 3 h (second reaction of Scheme **62**, *p.111*). The product precipitates during the reaction and it was filtrated and washed with water and acetone to afford **178** as a white solid (0.24 g, 89%).



^{1}H NMR (400 MHz, DMSO): δ 7.79 (d, *J* = 8.5 Hz, 2H, 2xC<u>H</u>$_{arom}$), 6.90 (d, *J* = 8.5 Hz, 2H, 2xC<u>H</u>$_{arom}$), 3.94 (t, *J* = 6.3 Hz, 2H, OC<u>H</u>$_2$CH$_2$), 2.38 (s, 3H COC<u>H</u>$_3$), 2.27 (t, *J* = 7.2 Hz, 2H, COC<u>H</u>$_2$CH$_2$), 1.86 – 1.81 (m, 2H, CH$_2$C<u>H</u>$_2$CH$_2$).

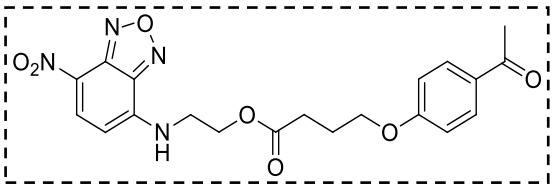
^{13}C NMR (101 MHz, DMSO): δ 196.47 (s, C$_{arom}$<u>C</u>OCH$_3$), 174.20 (s, O<u>C</u>OCH$_2$), 162.48 (s, CH<u>C</u>$_{arom}$OCH$_2$), 130.61 (s, <u>C</u>H$_{arom}$), 129.92 (s, CH<u>C</u>$_{arom}$COCH), 114.34 (s, <u>C</u>H$_{arom}$), 67.08 (s, O<u>C</u>H$_2$CH$_2$), 30.11 (s, CO<u>C</u>H$_2$CH$_2$), 26.50 (s, CO<u>C</u>H$_3$), 24.18 (s, CH$_2$<u>C</u>H$_2$CH$_2$).

HRMS (ESI): *m/z* calculated [M+Na]$^+$ = 245.078430, found [M+Na]$^+$ = 245.077406.

- Synthesis of compound **179**

Literature Basis – Liu, Z.; Hu, J.; Sun, J.; He, G.; Li, Y.; Zhang, G.; *Journal of Polymer Science Part A: Polymer Chemistry* **2010,** *48 (16)*, 3573

In a round bottom flask, under inert atmosphere, **178** (25 mg, 0.11 mmol), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 65 mg, 0.34 mmol) and 4-dimethylaminopyridine (DMAP, 2 mg, 0.01 mmol) were mixed at 0 ºC in 1 mL of previously distilled dichloromethane and stirred for 1 h. A solution of NBDNH(CH$_2$)$_2$OH (**163**, 50 mg, 0.23 mmol) in 0.5 mL of previously distilled acetone was added *via* a syringe and the solution was stirred for 5 h at room temperature (third reaction of Scheme **62**, *p.111*). Then, the solvent was concentrated *in vacuo* and the residue was dissolved in dichloromethane and washed with a solution of 5% NaHCO$_3$. The organic phases were dried (MgSO$_4$) and concentrated to a residue that was further purified by column chromatography (silica gel, dichloromethane:ethyl acetate 1:0 – 9:1) to afford **179** as an orange solid (34 mg, 71%).



^{1}H NMR (400 MHz, DMSO): δ 9.44 (s, 1H, CNHCH$_2$), 8.44 (d, *J* = 9.0 Hz, 1H, CHCH$_{arom}$CNO$_2$), 7.78 (d, *J* = 8.6 Hz, 2H, CH$_{arom}$), 6.85 (d, *J* = 8.6 Hz, 2H, CH$_{arom}$), 6.41 (d, *J* = 9.0 Hz, 1H, NHCCH$_{arom}$CH), 4.27 (t, *J* = 5.0 Hz, 2H, NHCH$_2$CH$_2$O), 3.95 (t, *J* = 6.3 Hz, 2H, OCH$_2$CH$_2$), 3.70 (s, 2H, NHCH$_2$CH$_2$O), 2.44 (s, 3H, COCH$_3$), 2.40 (t, *J* = 7.1 Hz, 2H, COCH$_2$CH$_2$), 1.92 – 1.87 (m, 2H, CH$_2$CH$_2$CH$_2$).

^{13}C NMR (101 MHz, DMSO): δ 196.26 (s, CCOCH$_3$), 172.56 (s, OCOCH$_2$), 162.22 (s, CHC$_{arom}$OCH$_2$), 145.21 (s, 2xC$_{arom}$NO), 144,43 (s, CC$_{arom}$NO$_2$CH), 144,07 (s, CC$_{arom}$NHCH$_2$), 137.88 (s, CHCH$_{arom}$CNO$_2$), 130.42 (s, CH$_{arom}$), 129.82 (s, CHC$_{arom}$COCH), 114.10 (s, CH$_{arom}$), 99.54 (s, NHCCH$_{arom}$CH), 66.86 (s, OCH$_2$CH$_2$), 61.79 (s, NHCH$_2$CH$_2$O), 42.31 (s, NHCH$_2$CH$_2$O), 30.09 (s, COCH$_2$CH$_2$), 26.41 (s, COCH$_3$), 24.00 (s, CH$_2$CH$_2$CH$_2$).

HRMS (ESI): *m/z* calculated [M+Na]$^+$ = 451.122420, found [M+Na]$^+$ = 451.121982.

- Conjugation of compound **179** with Lysozyme

Compound **179** dissolved in DMSO (200 μM) reacted with 10 μM of lysozyme in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes of reaction the solution was evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. HRMS results of deconvulted masses for unconjugated and conjugated species are shown in Table **28**, Figure **56** and Figure **57**.

Table **28** – Conjugation of compound **179** with Lysozyme

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Lysozyme	14296.77072	[M+H]+	100.0	-
Lysozyme + ***179***	14687.05288	[M+H]$^+$	6.5	14687.01321

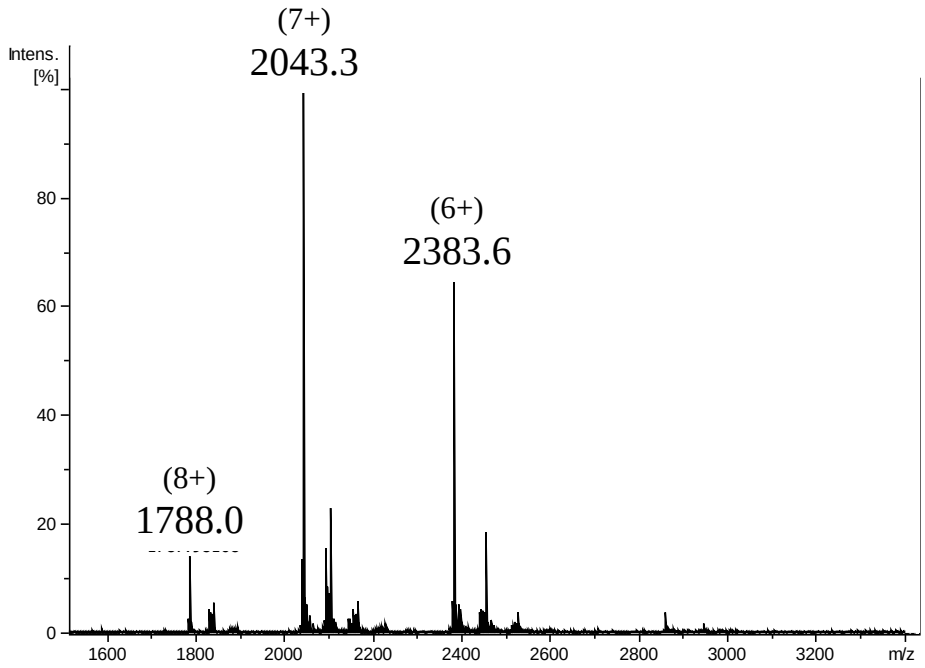


Figure **56** – Lyzosyme (10.0 µM) with compound **179** dissolved in DMSO (200.0 µM) in ammonium acetate buffer (50.0 mM, pH 7.0) after 30 minutes – Positive Mode of ESI-FTICR-MS full spectrum

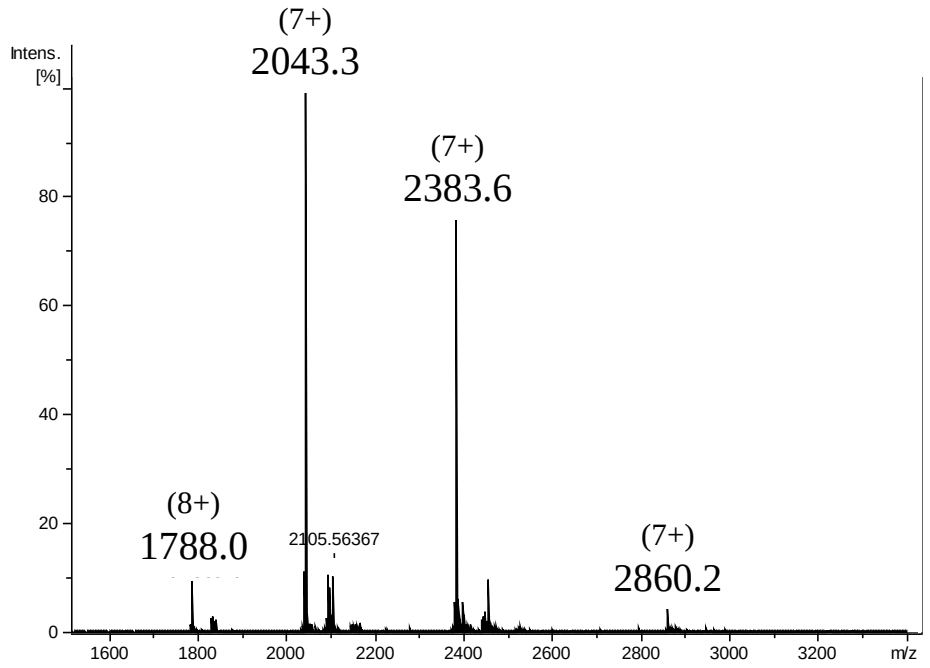


Figure **57** – Lyzosyme (10.0 µM) with compound **179** dissolved in DMSO (200.0 µM) in ammonium acetate buffer (50.0 mM, pH 7.0) after 2 h – Positive Mode of ESI-FTICR-MS full spectrum

- Bioimaging Cell Assays

Cell staining procedure: Cell lines were culture in RPMI-1640 medium with phenol red supplemented with 10% fetal bovine serum and antibiotic antimycotic solution (100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 mg/ml amphotericin B) at 37 ºC and under a 5% CO_2 atmosphere.

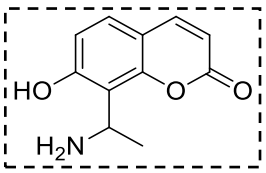
Cells were plated on a Slide 8 well plate and incubated for 2 days in the incubator. Prior to the addition of the cells the chambers were coated with poly-L-Lysine for one h and then each well washed with fresh cell culture medium. Samples to be tested were added at approximately 20 μM concentration in phenol-red free RPMI-1640 medium for 4 h. When pre-treatment with FA-ethylenediamine was employed, the concentration used was 71.4 μM and the incubation time was 1 h. Cells were washed and their membrane stained with WGA-Alexa 594 dye for 15-20 minutes. After this staining treatment, the cells were washed and fresh phenol-red free RPMI-1640 medium was added. Cell imaging was carried on a Leica TCS-SP5 Multiphoton/Confocal Fluorescence Microscope equipped with a continuous Ar ion laser (458, 476, 488, 496 and 514 nm) and a Helium-Neon laser (633 nm).

VI.4.3.2.2. *Cell release study (preliminary results)*

- Synthesis of compound **181**

Literature basis – Denton, T.T; Zhang, X.; Cashman, J.R. *Journal of Medicinal Chemistry,* **2005***, 48 (1),* 224

In a round bottom flask, under inert atmosphere, compound **183** (50 mg, 0.25 mmol), ammonium acetate (**184**, 0.2 g, 2.45 mmol) and molecular sieves (30% (m/m)) were mixed at room temperature in 1 mL of previously distilled methanol. Then, sodium cyanoborhydride (30 mg, 0.49 mmol) was added to the reaction mixture and the resultant solution was stirred at room temperature for 15 h (Scheme **64**, *p.112*). Then, a filtration was performed to remove the molecular sieves and the solvent was concentrated *in vacuo* to be further purified through recryrstalization with acetone/diethyl ether and acetone/hexane. Compound **171** was obtained as a white greenish solid (17 mg, 30%).



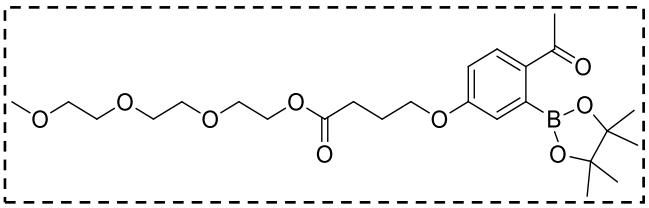
^{1}H NMR (400 MHz, DMSO) δ 7.80 (d, *J* = 9.3 Hz, 1H, -CC<u>H</u>CHCO-), 7.27 (d, *J* = 8.6 Hz, 1H, C<u>H</u>$_{arom}$), 6.44 (d, *J* = 8.6 Hz, 1H, C<u>H</u>$_{arom}$), 5.94 (d, *J* = 9.3 Hz, 1H, -CCHC<u>H</u>CO-), 4.71 (dd, *J* = 13.3, 6.6 Hz, 1H, -CC<u>H</u>NH_2CH_3), 1.38 (d, *J* = 6.7 Hz, 3H, -CCHNH_2C<u>H</u>$_3$).

VI.4.4. Experimental Details of Therapeutics

VI.4.4.1. PEGylation of biomolecules

- Synthesis of pinacolyl boronate ester of **186** (**222**c)

Following the general procedure for esterification of compound **141**, using (triethyleneglycol)monomethyl ester (**185**) as the alcohol derivative (0.6 mmol) and DCM instead of ACN, after 24 h, compound **222**c was obtained as a colorless oil in 70% yield (0.1 g).



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, *J* = 8.6 Hz, 1H, C<u>H</u>$_{arom}$), 6.91 (s, 1H, C<u>H</u>$_{arom}$), 6.81 (dd, *J* = 8.6, 1.5 Hz, 1H, C<u>H</u>$_{arom}$), 4.22 – 4.17 (m, 2H, -OC<u>H</u>$_2$-), 4.03 (t, *J* = 6.0 Hz, 2H, OC<u>H</u>$_2$CH$_2$), 3.67 – 3.63 (m, 2H, -OC<u>H</u>$_2$-), 3.63 – 3.56 (m, 6H, -OC<u>H</u>$_2$-), 3.50 (dd, *J* = 6.0, 3.0 Hz, 2H, -OC<u>H</u>$_2$-), 3.32 (d, *J* = 0.6 Hz, 3H, CH$_2$OC<u>H</u>$_3$), 2.54 – 2.48 (m, 5H, CH$_2$C<u>H</u>$_2$CH$_2$ and COC<u>H</u>$_3$), 2.11 – 2.05 (m, 2H, CH$_2$C<u>H</u>$_2$COO), 1.39 (s, 12H, CC<u>H</u>$_3$).

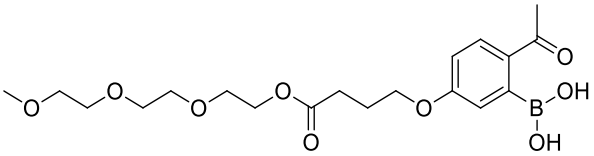
^{13}C NMR (101 MHz, $CDCl_3$) δ 198.40 (s, C$_{arom}$<u>C</u>OCH$_3$), 173.00 (s, CH$_2$<u>C</u>OO), 162.49 (s, <u>C</u>$_{arom}$OCH$_2$), 133.44 (s, CH$_3$CO<u>C</u>$_{arom}$), 130.75 (s, <u>C</u>H$_{arom}$), 117.87 (s, <u>C</u>H$_{arom}$), 113.97 (s, <u>C</u>H$_{arom}$), 83.56 (s, <u>C</u>(CH$_3$)$_2$), 71.89 (s, -OC<u>H</u>$_2$-), 70.57 (s, -OC<u>H</u>$_2$-), 70,53 (s, -OC<u>H</u>$_2$-), 69.06 (s, -OC<u>H</u>$_2$-), 66.77 (s, O<u>C</u>H$_2$CH$_2$), 63.62 (s, -OC<u>H</u>$_2$-), 59.02 (s CH$_2$O<u>C</u>H$_3$), 30.42 (s, CH$_2$<u>C</u>H$_2$CH$_2$), 24.94 (s, C(<u>C</u>H$_3$)$_2$), 24,82 (s, CO<u>C</u>H$_3$), 24.39 (s, CH$_2$<u>C</u>H$_2$CO).

^{13}C-HMBC 138.4 (s, C$_{arom}$B)

HRMS (ESI): *m/z* calculated $[M+H]^+$ = 495.27644, found $[M+H]^+$ = 495.27638.

- Synthesis of compound **186**

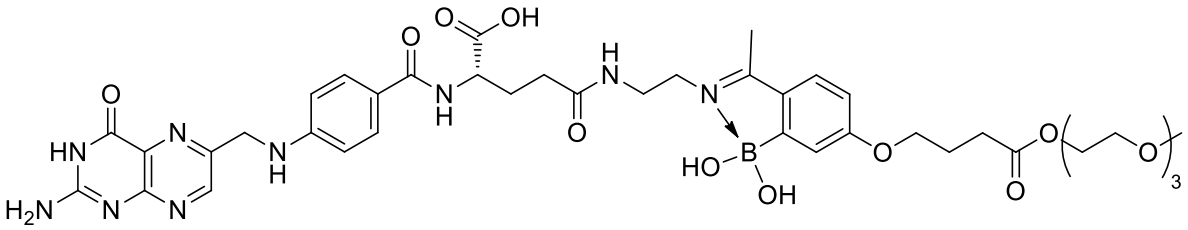
Following the general procedure of deprotection of pinacolyl boronate ester and using compound **222**c as the starting material (90 mg, 0.2 mmol), after 11 h, compound **186** was obtained as a colorless oil in 50% yield (39 mg).



HRMS (ESI): *m/z* calculated $[M+Na]^+$ = 435.18004, found $[M+Na]^+$ = 435.18012

- Conjugation of **186** with EDA-FA

Compounds **186** was dissolved in DMSO, as well as EDA-FA. From these solutions compound **186** (100 µM) reacted with 100 µM of EDA-FA in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes, the reactions mixtures were evaluated by performing an ESI-FTICR-MS and the conjugated species were detected.



HRMS (ESI): *m/z* calculated $[M+H]^+$ = 878.39833, found $[M+H]^+$ = 878.39054

- Conjugation (and reversibility) of compound **186** with Insulin

Compound **186** dissolved in DMSO (200 µM) reacted with 10 µM of insulin in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes of reaction the solution was evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. HRMS results of deconvulted masses for unconjugated and conjugated species are shown in Table **29**, alongside with the relative abundances of the species.

Table **29** – Conjugation of compound **186** with Insulin

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Insulin	5804.67089	$[M+H]^+$	100.0	-
Insulin + ***186***	6198.84587	$[M+H]^+$	40.6	6198.85059
Insulin + *2x**186***	6593.01857	$[M+H]^+$	23.7	6593.03029
Insulin + *3x**186***	6987.18817	$[M+H]^+$	10.1	6987.20999

The reversibility of the reaction was studied by removing 240 µL of the evaluated reaction and mixing it with 10 µL of an aqueous solution of D-fructose (0.2 M) to perform another ESI-FTICR-MS and detect the abundance of the conjugates. Table **30** depicts the decrease of both conjugated species in terms of relative abundance.

Table **30** – Conjugation of compound **186** with Insulin after adding 1 equivalent of D-fructose (8 mM)

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Insulin	5804.67089	$[M+H]^+$	100.0	-
Insulin + ***186***	6198.86322	$[M+H]^+$	25.5	6198.871333
Insulin + 2x***186***	6593.04273	$[M+H]^+$	21.7	6593.051036
Insulin + 3x***186***	6987.194965	$[M+H]^+$	11.1	6987.230739

Then, the conjugation system was further stressed by removing 100 µL of the previous evaluated reaction and mixed it with 40 µL of an aqueous solution of D-fructose (0.2 M) to perform another ESI-FTICR-MS and detect the abundance of the conjugates. Table **31** depicts the decrease of both conjugated species in terms of relative abundance.

Table **31** – Conjugation of compound **186** with Insulin after adding 10 equivalent of D-fructose (50 mM)

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Insulin	5804.67163	$[M+H]^+$	100.0	-
Insulin + ***186***	6198.854375	$[M+H]^+$	15.1	6198.851333
Insulin + 2x***186***	6593.023645	$[M+H]^+$	9.8	6593.031036
Insulin + 3x***186***	6987.194475	$[M+H]^+$	5.1	6987.210739

- Conjugation of **186** with Lysozyme

Compound **186** dissolved in DMSO (200 µM) reacted with 10 µM of lysozyme in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes of reaction the solution was evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. HRMS results of deconvulted masses for unconjugated and conjugated species are shown in Table **32**.

Table **32** – Conjugation of **186** with Lysozyme

Reaction	***Deconvoluted Mass***	***Molecule***	***Abundance [%]***	***Expected Mass***
Lysozyme	14296.77789	$[M+H]^+$	100.0	
Lysozyme + ***186***	14690.96088	$[M+H]^+$	11.4	14690.95759

- Conjugation of **186** with Lanreotide

Compound **186** dissolved in DMSO (200 µM) reacted with 10 µM of lanreotide in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes of reaction the solution was evaluated by

performing an ESI-FTICR-MS and the conjugated species were detected. HRMS results of deconvulted masses for unconjugated and conjugated species are shown in Table **33**.

Table **33** – Conjugation of compound **186** with Lanreotide

Reaction	*Deconvoluted Mass*	*Molecule*	*Abundance [%]*	*Expected Mass*
Lanreotide	1096.48289	$[M+H]^+$	100.0	-
Lanreotide + ***186***	1508.68336	$[M+H]^+$	8.7	1508.67316
Lanreotide + ***186***	1491.67312	$[M+H-H_2O]^+$	8.4	1491.67042
Lanreotide + ***2x186***	1919.87037	$[M+H]^+$	5.6	1919.85615
Lanreotide + ***2x186***	1901.85868	$[M+H-H_2O]^+$	1.5	1901.84613

- Conjugation of **186** with Somatostatin

Compound **186** dissolved in DMSO (200 µM) reacted with 10 µM of somatostatin in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes of reaction the solution was evaluated by performing an ESI-FTICR-MS and the conjugated species were detected. HRMS results of deconvulted masses for unconjugated and conjugated species are shown in Table **34**.

Table **34** – Conjugation of compound **186** with Somatostatin

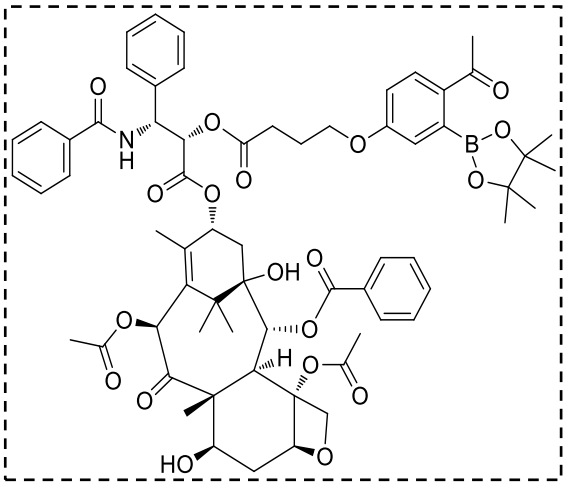
Reaction	*Deconvoluted Mass*	*Molecule*	*Abundance [%]*	*Expected Mass*
Somatostatin	1637.79955	$[M+H]^+$	91.2	-
Somatostatin + ***186***	2031.01289	$[M+H-H_2O]^+$	3.8	2030.97980
Somatostatin + ***186***	2014.00736	$[M+H-2H_2O]^+$	17.8	2013.96978
Somatostatin + ***2x186***	2443.27939	$[M+H-H_2O]^+$	2.0	2443.16274
Somatostatin + ***2x186***	2425.24118	$[M+H-2H_2O]^+$	2.9	2425.15273
Somatostatin + ***2x186***	2407.21567	$[M+H-3H_2O]^+$	2.8	2407.14271

VI.4.4.2. Study of the selectivity of internalization of iminoboronate conjugates

- Synthesis of pinacolyl boronate ester of **188** (**222**d)

Following the general procedure for esterification of compound **141**, using paclitaxel (**187**) as the alcohol derivative (0.6 mmol), EDC instead of DCC and DCM instead of ACN, after 24 h, compound **222**d was obtained as a white solid in 81% yield (56 mg).

Literature basis for NMR characterization of PTX – Chen, J.-Z.; Ranade, S.V.; Xie, X.-Q. *International Journal of Pharmaceutics* **2005**, *305*, 129 and Chmurny, G.N.; Hilton, B.D.; Brobst, S.; Look, S.A.; Witherup, K.M.; Beutler, J.A. *Journal of Natural Products* **1992**, *55(4),* 414



^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, *J* = 8.0 Hz, 2H, 2xC<u>H</u>$_{arom}$), 7.73 (dd, *J* = 13.6, 8.1 Hz, 3H, 3xC<u>H</u>$_{arom}$), 7.60 (t, *J* = 7.3 Hz, 1H, C<u>H</u>$_{arom}$), 7.50 (dd, *J* = 14.6, 7.3 Hz, 3H, 3xC<u>H</u>$_{arom}$), 7.41 – 7.30 (m, 7H, 7xC<u>H</u>$_{arom}$), 6.91 (dd, *J* = 17.2, 5.4 Hz, 2H, N<u>H</u> and C<u>H</u>$_{arom}$), 6.81 (dd, *J* = 8.6, 1.8 Hz, 1H, C<u>H</u>$_{arom}$), 6.37 – 6.12 (m, 2H, -C<u>H</u>(OCOCH$_3$)- and C(CH$_3$)C<u>H</u>(OCO)CH$_2$), 6.01 – 5.93 (m, 1H, -NHC<u>H</u>(Ph)CH(OCO)-), 5.67 (d, *J* = 7.0 Hz, 1H, -C(OH)C<u>H</u>(OCOPh)CH-), 5.53 (d, *J* = 2.8 Hz, 1H, -CH(Ph)C<u>H</u>(OCO)COO-), 4.96 (d, *J* = 9.5 Hz, 1H, -CH$_2$C<u>H</u>(OCH$_2$)C-), 4.44 (dd, *J* = 10.7, 6.7 Hz, 1H, -C(CH$_3$)C<u>H</u>(OH)CH$_2$-), 4.31 (d, *J* = 8.5 Hz, 1H, C(OCOCH$_3$)C<u>H$_{2a}$</u>OCH), 4.19 (d, *J* = 8.4 Hz, 1H, C(OCOCH$_3$)C<u>H$_{2b}$</u>OCH), 4.05 – 3.96 (m, 2H, -OC<u>H$_2$</u>CH$_2$CH$_2$-), 3.81 (d, *J* = 7.0 Hz, 1H, -CH(OCOPh)C<u>H</u>C(OCOCH$_3$)-), 2.71 – 2.52 (m, 6H, -CH$_2$C<u>H$_2$</u>COO- and -C(OH)C<u>H$_{2b}$</u>C(OCH$_2$)- and C$_{arom}$COC<u>H$_3$</u>), 2.46 (s, 3H, -COC<u>H$_3$</u>), 2.37 (dd, *J* = 15.2, 9.3 Hz, 1H, -CH(OCO)C<u>H$_{2a}$</u>C(OH)-), 2.21 (s, 3H, -COC<u>H$_3$</u>), 2.18 – 2.02 (m, 5H, -CH(OCO)C<u>H$_{2b}$</u>C(OH)- and -CH$_2$C<u>H$_2$</u>CH$_2$- and 2xC(O<u>H</u>)), 1.94 (s, 3H, -COCC<u>H$_3$</u>CH(OH)-), 1.91 – 1.83 (m, 1H, -C(OH)C<u>H$_{2a}$</u>C(OCH$_2$)-), 1.67 (s, 3H, C=CC<u>H$_3$</u>), 1.43 (s, 12H, 2xOC(C<u>H$_3$</u>)$_2$), 1.22 (s, 3H, 1xC(C<u>H$_3$</u>)$_2$), 1.12 (s, 3H, 1xC(C<u>H$_3$</u>)$_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 203.90 (s, C(OCOCH$_3$)<u>C</u>OC(CH$_3$)), 198.56 (s, C$_{arom}$<u>C</u>OCH$_3$), 172.21 (s, -CH(OCO)<u>C</u>OOCH-), 171.35 (s, -CHO<u>C</u>OCH$_3$), 169.90 (s, -CO<u>C</u>OCH$_3$), 168.14 (s, -CH$_2$<u>C</u>OO-), 167.20 (s, Ph<u>C</u>OO-), 167.10 (s, Ph<u>C</u>ONH-), 162.40 (s, <u>C</u>$_{arom}$OCH$_2$), 142.81 (s, -CH(OCO)<u>C</u>(CH$_3$)C-), 136.94 (s, <u>C</u>$_{arom}$CONH), 133.78 (s, <u>C</u>H$_{arom}$), 133.74 (s, <u>C</u>$_{arom}$CHNH), 133.65 (s, C$_{arom}$<u>C</u>OCH$_3$), 132.88 (s, -C(CH$_3$)<u>C</u>C(CH$_3$)$_2$C(OCOCH$_3$)-), 132.15 (s, <u>C</u>H$_{arom}$), 130.86 (s, <u>C</u>H$_{arom}$), 130.32 (s, <u>C</u>H$_{arom}$), 129.26 (s, <u>C</u>$_{arom}$COOCH), 129.20 (s, <u>C</u>H$_{arom}$), 128.86 (s, <u>C</u>H$_{arom}$), 128.63 (s, <u>C</u>H$_{arom}$), 127.15 (s, <u>C</u>H$_{arom}$), 126.55 (s, <u>C</u>H$_{arom}$), 118.10 (s, <u>C</u>H$_{arom}$), 113.94 (s, <u>C</u>H$_{arom}$), 84.53 (s, -CH$_2$<u>C</u>H(OCH$_2$)C-), 83.74 (s, <u>C</u>(CH$_3$)$_2$), 81.13 (s, <u>C</u>H(OCOCH$_3$), 79.21 (s, -CH$_2$<u>C</u>(OH)CH(OCOPh)-), 76.52 (s, C(OCOCH$_3$)<u>C</u>H$_2$OCH), 75.68 (s, <u>C</u>H(OCOCH$_3$), 75.17 (s, -C(OH)<u>C</u>H(OCOPh)CH-), 74.13 (s, -CH(Ph)<u>C</u>H(OCO)COO-), 72.22 (s., C(CH$_3$)<u>C</u>H(OCO)CH$_2$), 71.95 (s, -C(CH$_3$)<u>C</u>H(OH)CH$_2$-), 66.46 (s, -O<u>C</u>H$_2$CH$_2$-), 58.58 (s, -COC<u>C</u>H$_3$CH(OH)-), 52.81 (s, -NH<u>C</u>H(Ph)CH(OCO)-), 45.66 (s, -CH(OCOPh)<u>C</u>HC(OCOCH$_3$)-), 43.26 (s, C$_{sp2}$<u>C</u>(CH$_3$)$_2$C(OH)-), 35.66 (s, -C(OH)<u>C</u>H$_2$C(OCH$_2$)-), 35.62 (s, -CH(OCO)<u>C</u>H$_2$C(OH)-), 30.22

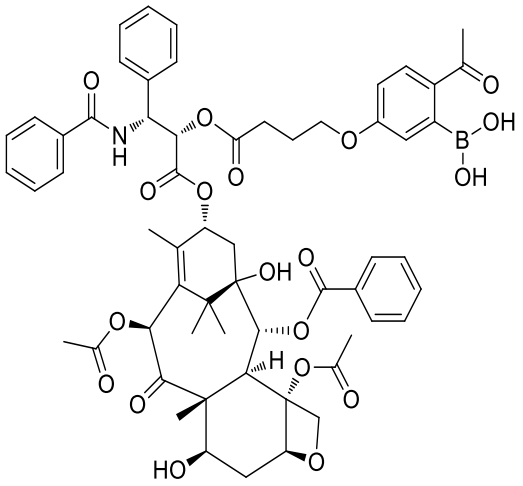
(s, -$CH_2\underline{C}H_2CO$-), 26.89 (s, -$C(\underline{C}H_3)_2$), 25.05 (s, -$OC(\underline{C}H_3)_2$), 24.93 (s, $C_{arom}CO\underline{C}H_3$), 24.34 (s, -$CH_2\underline{C}H_2CH_2$-), 22.82 (s, -$OCOC\underline{H}_3$), 22.24 (s, -$C(\underline{C}H_3)_2$), 20.94 (s, -$OCOC\underline{H}_3$), 14.95 (s, $C(\underline{C}H_3)$), 9.70 (s, $C=C\underline{C}H_3$).

^{13}C-HMBC 138.2 (s, $\underline{C}_{arom}B$)

HRMS (ESI): *m/z* calculated $[M+K]^+$ = 1222.45903, found $[M+K]^+$ = 1222.46159

- Synthesis of compound **188**

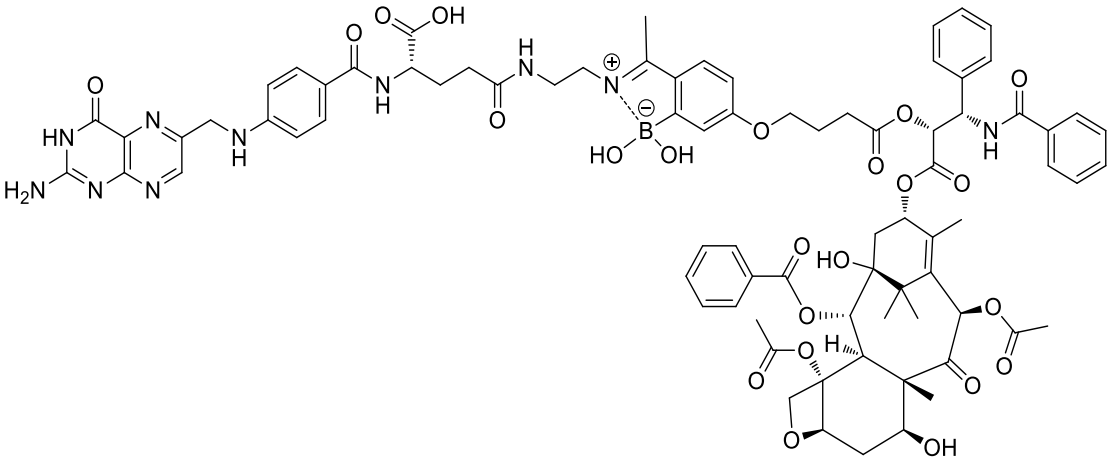
Following the general procedure of deprotection of pinacolyl boronate ester and using compound **222**d as the starting material (56 mg, 0.05 mmol), after 6 h, compound **188** was obtained as a white solid in 67% yield (34 mg).



HRMS (ESI): *m/z* calculated $[M+Na]^+$ = 1124.40676, found $[M+Na]^+$ = 1124.40796

- Conjugation of **188** with EDA-FA

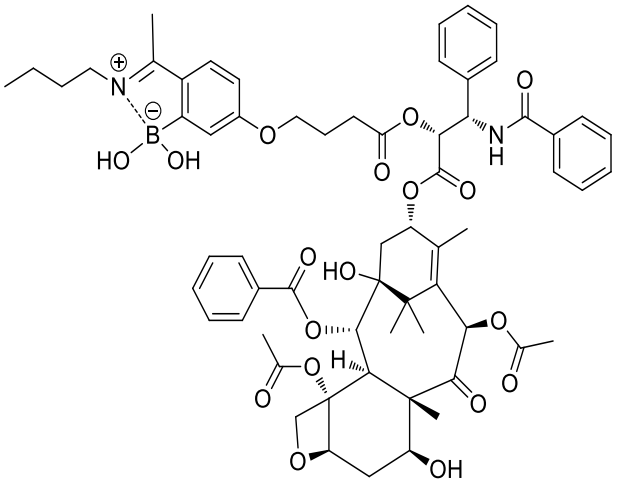
Compounds **188** was dissolved in DMSO, as well as EDA-FA. From these solutions compound **188** (100 µM) reacted with 100 µM of EDA-FA in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes, the reactions mixtures were evaluated by performing an ESI-FTICR-MS and the conjugated species were detected:



HRMS (ESI): *m/z* calculated $[M+H]^+$ = 1567.62499, found $[M+H]^+$ = 1567.62473

- Conjugation of **188** with 1-butylamine

Compounds **188** was dissolved in DMSO, as well as 1-butylamine. From these solutions compound **188** (100 µM) reacted with 100 µM of 1-butylamine in $NH_4CH_3CO_2$ buffer (50.0 mM, pH 7.0) at room temperature. After 10 minutes, the reactions mixtures were evaluated by performing an ESI-FTICR-MS and the conjugated species were detected:



HRMS (ESI): *m/z* calculated $[M+H]^+$ = 1157.50346, found $[M+H]^+$ = 1157.50685

- Toxicity and Viability Cell Assays

Cell Culture. Human normal skin fibroblasts (CRL-1502), colon adenocarcinoma (CACO-2) and lung cancer (NCI-H460) cell lines were purchased from ATCC and cultivated in media RPMI-1640 with L- glutamine and supplemented with 10% fetal bovine serum (FBS), antibiotic and antimycotic solution and kept in a humidified atmosphere with 5% CO_2 and at 37 ºC.

Anti-proliferative assay. NCI-H460 cells were plated in 96-well plates at a density of $5x10^4$ cells/mL. Plates were incubated overnight and treated next day with the samples to be tested. Samples comprised compound **188** (ABBA PTX derivative), compound **170** (EDA-FA), conjugate **189** (between ABBA PTX derivative and EDA-FA) and conjugate between compound **188** and 1-butylamine. These last conjugates were obtained by mixing equal micromolar amounts of compound **188** with either compound **170** or 1-butylamine and leaving mixtures at room temperature for approximately 1 h. These samples were then diluted with the cell culture media with only 0.5% FBS (after dilution, the percentage of organic solvent is <<1% (v/v)). Cells were incubated for 48 h with several concentrations of the samples to be tested. After incubation, media was removed and cells washed with Hank´s balanced salt solution (HBSS). 0.5% FBS fresh cell culture media containing 50 g/ml neutral red was added to the plate and left for 3 h. Cells were then washed with PBS and the amount of neutral red retained by the cells extracted and dissolved with an organic solution (19.96 ml distilled water, 20 ml ethanol and 400 l glacial acetic acid). Plate was gently shake and read at 540 nm in a plate reader. Viability was determined by the ratio of absorbance of treated cells and non-treated cells (control). For each experimental condition, 4 repliques were done. IC_{50} were determined using GraphPad Prism software.

Toxicity Assays. CRL-1502 cells were plated in 96-well plates and grown until forming a monolayer. Samples were prepared as described previously. In this case, incubation was carried on for 24 h and viability was determined likewise as explained before. Results are shown in Figure **42** (*p.118*) and Figure **58**.

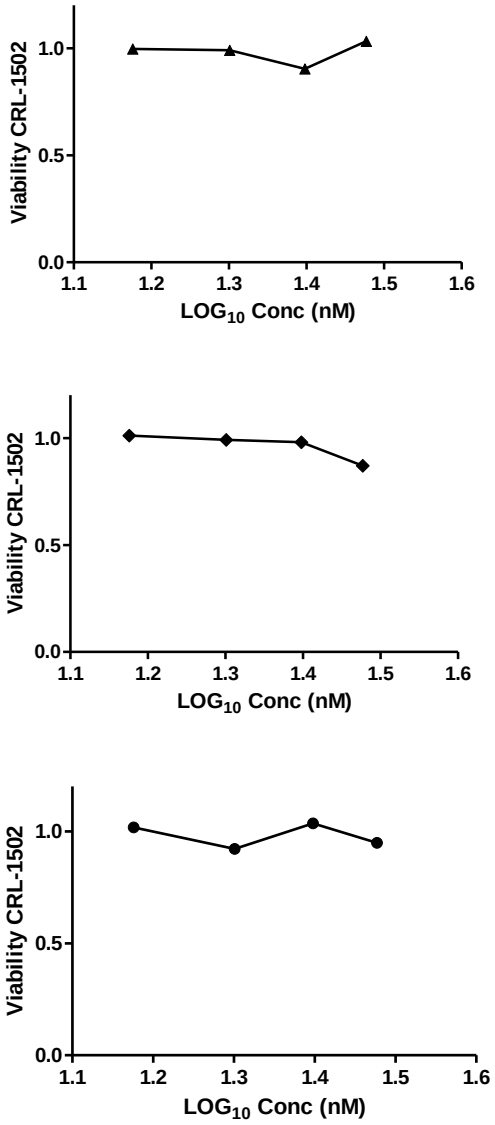


Figure **58** – Viability assay for CRL-1502 (Human Skin Normal Fibroblast) cell model - 24 h of incubation

VI.5. Experimental Section of Chapter V

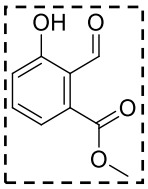
VI.5.1. Experimental Details of pH Controlled Irreversibility

VI.5.1.1. Synthesis of irreversible molecule

- Synthesis of compound **197**

Literature basis – Gleye, C.; Lewin, G.; Laurens, A.; Jullian, J.-C.; Loiseau, P.; Bories, C.; Hocquemiller, R. *Journal of Natural Products* **2003**, *66(5)*, 690 and Sato, M.; Kuwahara, Y.; Matsuyama, S.; Suzuki, T.; *Bioscience, Biotechnology and Biochemistry* **1993**, *57(8)*, 1299

In a round bottom flask, compound **198** (0.5 g, 3.6 mmol) and hexamethylenetetramine (0.54 g, 3.6 mmol) were mixed in 10 mL of trifluoroacetic acid was heated at reflux for 6 h (first reaction of Scheme **70**, *p.126*). The reaction mixture was diluted with water and extracted with diethyl ether, dried over Na_2SO_4, and filtered and solvent was evaporated under reduced pressure. The crude product was then purified by flash column chromatography (silica gel, petroleum ether:dichloromethane:ether 3:1:1) to afford **197** as a white solid (0.3 g, 50%).



^{1}H NMR (600 MHz, $CDCl_3$) δ 12.19 (s, 1H, $C_{arom}O\underline{H}$), 10.62 (s, 1H, $C_{arom}CO\underline{H}$), 7.54 – 7.50 (m, 1H, $C\underline{H}_{arom}$), 7.46 (dd, *J* = 7.5, 1.1 Hz, 1H, $C\underline{H}_{arom}$), 7.15 (d, *J* = 8.4 Hz, 1H, $C\underline{H}_{arom}$), 3.94 (s, 3H, $C_{arom}COOC\underline{H}_3$).

^{13}C NMR (151 MHz, $CDCl_3$) δ 197.56 (s, $C_{arom}\underline{C}OH$), 166.38 (s, $C_{arom}\underline{C}OOCH_3$), 163.21 (s, $\underline{C}_{arom}OH$), 135.99 (s, $\underline{C}H_{arom}$), 133.58 (s, $\underline{C}_{arom}COOCH_3$), 122.65 (s, $\underline{C}H_{arom}$), 122.36 (s, $\underline{C}H_{arom}$), 118.37 (s, $\underline{C}_{arom}COH$), 52.95 (s, $C_{arom}COO\underline{C}H_3$).

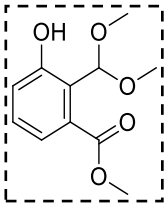
NMR characterization in accordance with the one described in literature

- Synthesis of compound **201**

Literature basis – Patwardhan, S. A.; Dev, S. *Synthesis* **1974**, 348; Sato, M.; Kuwahara, Y.; Matsuyama, S.; Suzuki, T.; *Bioscience, Biotechnology and Biochemistry* **1993**, *57(8)*, 1299 and Taylor, E.C.; Chiang, C.-S. Synthesis **1977** *(07)*, 467

In a round bottom flask, compound **197** (1 g, 5.5 mmol) and Amberlyst® 15 (25% (m/m)) were mixed with trimethyl orthoformate (**200**, 3 mL, 11 mmol) at room temperature for 30 h (second reaction of Scheme **70**, *p.126*). The reaction mixture was then filterend, using dichloromethane to clean the resin and the resulting solution was cleaned with sodium bicarbonate (2x10 mL) and brine (2x 10 mL). This solution

was dried over $MgSO_4$ and filtered under vaccum. In the end, the solvent was evaporated under reduced pressure to afford **201** as a colorless oil (1.2 g, 94%).



^{1}H NMR (600 MHz, $CDCl_3$) δ 8.89 (s, 1H, $C_{arom}O\underline{H}$), 7.31 (dd, *J* = 7.6, 1.2 Hz, 1H, $C\underline{H}_{arom}$), 7.25 (t, *J* = 7.9 Hz, 1H, $C\underline{H}_{arom}$), 7.02 (dd, *J* = 8.2, 1.2 Hz, 1H, $C\underline{H}_{arom}$), 6.30 (s, 1H, $C_{arom}C\underline{H}(OCH_3)_2$), 3.87 (s, 3H, $C_{arom}COOC\underline{H}_3$), 3.44 (s, 6H, $C_{arom}CH(OC\underline{H}_3)_2$).

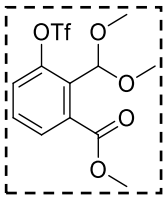
^{13}C NMR (151 MHz, $CDCl_3$) δ 168.10 (s, $C_{arom}\underline{C}OOCH_3$), 157.18 (s, $\underline{C}_{arom}OH$), 131.16 (s, $\underline{C}_{arom}COOCH_3$), 129.92 (s, $\underline{C}H_{arom}$), 121.73 (s, $\underline{C}H_{arom}$), 121.42 (s, $\underline{C}H_{arom}$), 120.84 (s, $\underline{C}_{arom}CH(OCH_3)_2$), 103.77 (s, $C_{arom}\underline{C}H(OCH_3)_2$), 54.63 (s, $C_{arom}CH(O\underline{C}H_3)_2$), 52.42 (s, $C_{arom}COO\underline{C}H_3$).

NMR characterization in accordance with the one described in literature

- Synthesis of compound **205**

Literature basis - Luker, T.; Bonnert, R.; Paine, S.W.; Schmidt, J.; Sargent, C.; Cook, A.R.; Cook, A.; Gardiner, P.; Hill, S.; Weyman-Jones, C.; Patel, A.; Thom, S.; Thorne, P.; *Journal of Medicinal Chemistry* **2011** , *54 (6)*, 1779

In a round bottom flask, *N*-Phenyltrifluoromethanesulfonimide (**202** , 3.0 g, 8.4 mmol) was added portion wise to a solution of compound **201** (1.2 g, 5.3 mmol) and triethylamine (2.2 mL, 15.0 mmol) in DMF (13 mL) and the reaction stirred for 1 h (third reaction of Scheme **70**, *p.126*). Water was added and the mixture extracted with diethyl ether. The organic layer was washed with water, LiCl (aq), brine, dried (Na_2SO_4), filtrated, concentrated and purified by flash column chromatography (silica gel, hexane:diethyl ether (4:1 – 1:1)) to give **205** as a white solid (1.8 g, 95%).



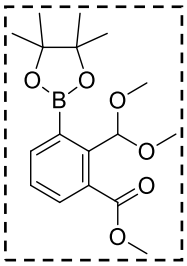
^{1}H NMR (600 MHz, $CDCl_3$) δ 7.59 – 7.51 (m, 1H, $C\underline{H}_{arom}$), 7.49 – 7.41 (m, 1H, $C\underline{H}_{arom}$), 7.34 (dd, *J* = 8.3, 1.2 Hz, 1H, $C\underline{H}_{arom}$), 5.81 (s, 1H, $C_{arom}C\underline{H}(OCH_3)_2$), 3.89 (s, 3H, $C_{arom}COOC\underline{H}_3$), 3.40 (s, 6H, $C_{arom}CH(OC\underline{H}_3)_2$).

^{13}C NMR (151 MHz, $CDCl_3$) δ 168.09 (s, $C_{arom}\underline{C}OOCH_3$), 148.03 (s, $\underline{C}_{arom}COTf$), 134.47 (s, $\underline{C}_{arom}CH(OCH_3)_2$), 130.82 (s, $\underline{C}_{arom}COOCH_3$), 130.32 (s, $\underline{C}H_{arom}$), 128.47 (s, $\underline{C}H_{arom}$), 124.42 (s, $\underline{C}H_{arom}$), 118.59 (q, *J* = 320.0 Hz, - $SO_2\underline{C}F_3$), 100.58 (s, $C_{arom}\underline{C}H(OCH_3)_2$), 54.99 (s, $C_{arom}CH(O\underline{C}H_3)_2$), 52.76 (s, $C_{arom}COO\underline{C}H_3$).

- Synthesis of compound **204**

Literature basis - Patent: Anacor Pharmaceuticals, Inc.; GlaxoSmithKline; US2010/256092; (**2010**); (A1) English; Ding, C.Z.; Zhang, Y.-K.; Li, X.; Liu, Y.; Zhang, S.; Zhou, Y.; Plattner, J.J.; Baker, S.J.; Liu, L.; Duan, M.; Jarvest, R.L.; Ji, J.; Kazmierski, W.M.; Tallant, M.D.; Wright, L.L.; Smith, G.K.; Crosby, R.M.; Wang, A.A.; Ni, Z.-J.; Zou. W.; Wright, J. *Bioorganic & Medicinal Chemistry Letters* **2010**, *20*, 7317 and Xia, Y.; Cao, K.; Zhou, Y.; Alley, M.R.K.; Rock, F.; Mohan, M.; Meewan, M.; Baker, S.J.; Lux, S.; Ding, C.Z.; Jia, G.; Kully, M.; Plattner, J.J. *Bioorganic & Medicinal Chemistry Letters* **2011**, *21*, 2533

In a flame dried round bottom flask, under inert atmosphere, compound **205** (1.8 g, 5.1 mmol), bis(pinacolato)diboron (**120**, 2.6 g, 10.1 mmol), [1,1'-bis(diphenylphosphino)ferrocene]palladium (II) chloride (0.4 g, 0.51 mmol) and sodium acetate (1.2 g, 15.1 mmol) were flushed 3 times with Argon. Then, 35 mL of previously distilled dioxane were added and degassed for 10 minutes with bubbling Argon. The reaction mixture was heated at 95 ºC for 4 days (fourth reaction of Scheme **70**, *p.126*), after which the solvent was concentrated in vaccum and the crude product purified by flash column chromatography (silica gel, hexanedichloromethane:diethyl ether (9:0.5:0.5 – 8:1:1)) to give compound **204** as a yellowish solid (0.3 g, 20%).



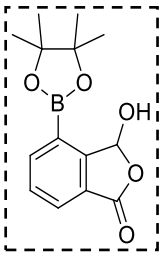
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, *J* = 7.7 Hz, 1H, C$\underline{H}_{arom}$), 7.52 (d, *J* = 7.2 Hz, 1H, C$\underline{H}_{arom}$), 7.33 (t, *J* = 7.5 Hz, 1H, C$\underline{H}_{arom}$), 6.08 (s, 1H, $C_{arom}C\underline{H}(OCH_3)_2$), 3.86 (s, 3H, $C_{arom}COOC\underline{H}_3$), 3.31 (s, 6H, $C_{arom}CH(OC\underline{H}_3)_2$), 1.36 (s, 12H, 2x$C(C\underline{H}_3)_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 168.78 (s, $C_{arom}\underline{C}OOCH_3$), 141.49 (s, $\underline{C}_{arom}B$), 135.27 (s, $\underline{C}H_{arom}$), 130.25 (s, $\underline{C}H_{arom}$), 129.79 (s, $\underline{C}_{arom}COOCH_3$), 127.68 (s, $\underline{C}H_{arom}$), 101.75 (s, $\underline{C}_{arom}CH(OCH_3)_2$), 83.53 (s, $\underline{C}(CH_3)_2$), 53.85 (s, $C_{arom}CH(O\underline{C}H_3)_2$), 52.22 (s, $C_{arom}COO\underline{C}H_3$), 25.09 (s, $C(\underline{C}H_3)_2$).

- Synthesis of compound **203**

Literature basis – Freskos, J.; Cynkowski, T.; Swenton J.S. *Chemical Communications*, **1984**, 819 and Griffin, D.R.; Kasko, A.M. *Journal of the American Chemical Society* **2012**, *134*, 13103

In a flame dried round bottom flask, under inert atmosphere, compound **204** (0.3 g, 0.89 mmol) was mixed with 2.5 mL of water. Then, 0.25 mL of trifluoroacetic acid were added and the reaction mixture was heated at 90 ºC for 18 h (fifth reaction of Scheme **70**, *p.126*). At this point, a white solid had precipitated and a filtration under reduced pressure was performed to isolate compound **203** (0.17 g, 67%).



^{1}H NMR (400 MHz, Acetone) δ 8.01 (d, *J* = 7.2 Hz, 1H, C<u>H</u>$_{arom}$), 7.94 – 7.80 (m, 1H, C<u>H</u>$_{arom}$), 7.64 (t, *J* = 7.4 Hz, 1H, C<u>H</u>$_{arom}$), 6.81 (s, 1H, C$_{arom}$C<u>H</u>O(OH)), 2.06 (s, 1H, C$_{arom}$CHO(O<u>H</u>)), 1.34 (s, 12H, 2xC(C<u>H</u>$_3$)$_2$).

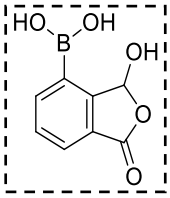
^{13}C NMR (101 MHz, Acetone) δ 141.14 (s, <u>C</u>H$_{arom}$), 130.78 (s, <u>C</u>H$_{arom}$), 127.81 (s, <u>C</u>H$_{arom}$), 85.21 (s, C$_{arom}$<u>C</u>HO(OH)), 25.38 (s, C(<u>C</u>H$_3$)$_2$).

Lack of solubility determined that C$_{quat}$ couldn't be detected**.**

- Synthesis of compound **206**

Literature basis – Pennington, T.E.; Kardiman, C.; Hutton, C.A.; *Tetrahedron Letters* **2004**, *45*, 6657

Treatment of compound **203** with 5 equiv of polystyrene–boronic acid (**146**) in acetonitrile/1 M HCl (9:1) for 20 h at 25 ºC (sixth reaction of Scheme **70**, *p.126*), yielded 48% of compound **206**, after evaporating solvents and performing a flash column chromatography (silica gel, hexane:acetone 1:9 – 0:1 and 2% MeOH).

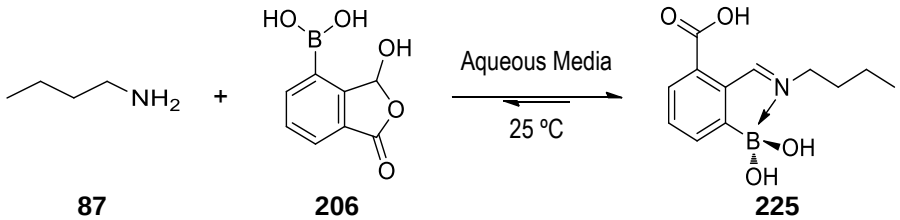


^{1}H NMR (400 MHz, D$_2$O) δ 7.99 (d, *J* = 7.3 Hz, 1H, C<u>H</u>$_{arom}$), 7.87 (d, *J* = 7.6 Hz, 1H, C<u>H</u>$_{arom}$), 7.63 (t, *J* = 7.5 Hz, 1H, C<u>H</u>$_{arom}$), 6.85 (s, 1H, C$_{arom}$C<u>H</u>O(OH)).

Lack of solubility determined that ^{13}C NMR was not able to be achieved yet

VI.5.1.2. Conjugation experiments with 1-butylamine

VI.5.1.2.1. *Preliminary reaction*



87 **206** **225**

Scheme **84** – Reaction between compound **206** and 1-butylamine

Compound **206** (17 mg) was added to a 10 mL round bottom flask and then dissolved in 0.4 mL of deuterated water. Afterwards, 1-butylamine (**87**, 9 µL, 0.09 mmol) was added to the same flask and these compounds reacted for 18 h at 25 ºC (Scheme **84**). At that time, the reaction mixture was transferred to a NMR tube and a ^{1}H-NMR spectrum was collected to evaluate the product's conversion based on the

comparison of the signal from *α*-protons of 1-butylamine and the same protons from imine/secondary amine. Evaluation of the ^{1}H-NMR spectrum demonstrated a 32% conversion into the imine.

VI.5.1.2.2. *Fructose addition*

Based on the experience with vicinal diols and the reversibility assays performed with iminoboronates 1 equivalent of D-fructose was added to the reaction mixture, in order to see whether or not the secondary amine formation could be catalysed. Unfortunately, the sugar completely hydrolysed the imine, but it is not clear that it boosted the cyclization. Further experiments on the subject have to be performed.

VI.5.1.2.3. *Influence of pH*

Compound **206** (6 mg) was added to an eppendorf and then dissolved in 0.15 mL of sodium acetate buffer (50.0 mM) at different pH's. Afterwards, 1 equivalent of 1-butylamine (**87**, 3 µL) was added and these compounds reacted for 18 h at 25 ºC (Scheme **84**). At that time, the reaction mixture was transferred to a NMR tube and diluted in D_2O to collect a ^{1}H-NMR spectrum and evaluate the product's conversion.

As a control, the same experiments following the same protocol were performed with a aboronated species (2-carboxybenzaldehyde) instead of compound **206,** to demonstrate that the boronic acid's presence is essential for imine formation.

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